Monoaminergic PET imaging and histopathological correlation in unilateral and bilateral 6-hydroxydopamine lesioned rat models of Parkinson's disease: a longitudinal *in-vivo* study

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Running title: ^{11}C-DTBZ PET in 6-OHDA rat models

Abstract

Carbon-11 labeled dihydrotetrabenazine (^{11}C-DTBZ) binds to the vesicular monoamine transporter 2 and has been used to assess nigro-striatal integrity in animal models and patients with Parkinson's disease. Here, we applied ^{11}C-DTBZ positron emission tomography (PET) to obtain longitudinally *in-vivo* assessment of striatal dopaminergic loss in the classic unilateral and in a novel bilateral 6-hydroxydopamine (6-OHDA) lesion rat model. Forty-four Sprague–Dawley rats were divided into 3 sub-groups: 1. 6-OHDA-induced unilateral lesion in the medial forebrain bundle, 2. Bilateral lesion by injection of 6-OHDA in the third ventricle, 3. Vehicle injection in either site. ^{11}C-DTBZ PET studies were investigated in the same animals successively at baseline, 1, 3 and 6 weeks after lesion using an anatomically standardized volumes-of-interest approach. Additionally, 12 rats had PET and Magnetic Resonance Imaging to construct a new ^{11}C-DTBZ PET template. Behavior was characterized by rotational, catalepsy and limb-use asymmetry tests and dopaminergic striatal denervation was validated post-mortem by immunostaining of the dopamine transporter (DAT). ^{11}C-DTBZ PET showed a significant decrease of striatal binding (SB) values one week after the unilateral lesion. At this point, there was a 60% reduction in SB in the affected hemisphere compared with baseline values in 6-OHDA unilaterally lesioned animals. A 46% symmetric reduction over baseline SB values was found in bilaterally lesioned rats at the first week after lesion. SB values remained constant in unilaterally lesioned rats whereas animals with bilateral lesions showed a modest (22%) increase in binding values at the 3rd and 6th week post-lesion. The degree of striatal dopaminergic denervation was corroborated histologically by DAT immunostaining. Statistical analysis revealed a high correlation between ^{11}C-DTBZ PET SB and striatal DAT immunostaining values ($r = 0.95$, $p<0.001$). The data presented here indicate that ^{11}C-DTBZ PET may be used to ascertain

changes occurring *in-vivo* throughout the evolution of nigro-striatal dopaminergic neurodegeneration, mainly in the unilateral 6-OHDA lesion rat.

Keywords: Parkinson's disease, 6-hydroxydopamine, Positron emission tomography, [^{11}C]-dihydrotetrabenazine (^{11}C-DTBZ), Nigro-striatal depletion, Dopaminergic marker, Rat models.

Abbreviations

3-D: Three-dimensional

6-OHDA: 6-hydroxydopamine

^{11}C-CH3I: Carbon-11 labeled methyliodide

^{11}C-DTBZ: Carbon-11 labeled dihydrotetrabenazine

ANOVA: Analysis of variance

BR: Binding ratio

DA: Dopamine

DAT: Dopamine transporter

DMSO: Dimethyl sulfoxide

KOH: Potassium hydroxide

MBq: Megabecquerel

MFB: Medial forebrain bundle

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

MRI: Magnetic resonance imaging

PD: Parkinson's disease

PET: Positron emission tomography

RAMLA: Row action maximum likelihood algorithm

SB: Striatal binding

SNpc: Substantia nigra pars compacta

SUV: Standardized uptake value

VMAT2: Vesicular monoamine transporter 2

VOI: Volume of interest

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder mainly characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and dopamine (DA) striatal depletion. Positron emission tomography (PET) imaging can be used for *in-vivo* assessment of the dopaminergic nigro-striatal projection (*Casteels et al., 2008*). Dopamine undergoes pre-synaptic reuptake through the dopamine transporter (DAT) protein and is packaged into synaptic vesicles by the vesicular monoamine transporter 2 (VMAT2). *In-vivo* and histological measurement of these transporters is used as indicator of presynaptic nerve terminal integrity. Among the available radioligands, [^{11}C]-dihydrotetrabenazine (^{11}C-DTBZ) specifically binds to VMAT2 and is currently considered to be a highly sensitive marker of striatal pre-synaptic dopaminergic activity in PD patients (*Frey et al., 1996; de la Fuente-Fernández et al., 2011*) and animal models (*Strome et al., 2006; Sossi et al., 2007; Collantes et al., 2008; Blesa et al., 2010*) and several studies have shown an excellent correlation between ^{11}C-DTBZ binding potential and histological nigro-striatal lesion (*Strome et al., 2006; Blesa et al., 2012; Brown et al., 2013*). However, there is no study evaluating the longitudinal changes in ^{11}C-DTBZ throughout the evolution of different lesion models. This is particularly interesting when considering the many changes that occur in the basal ganglia and elsewhere in the brain to compensate for initial dopaminergic depletion (*Zigmond et al., 1989; Bezard et al., 2001, 2003; Pifl and Hornykiewicz, 2006*). Here, we report the modification of ^{11}C-DTBZ PET in the unilateral 6-hydroxydopamine (6-OHDA) model, and in a novel bilaterally lesioned rat model using intraventricular injection of 6-OHDA.

Material and methods

Animals

Fifty-six Sprague–Dawley rats (300 g; Harlan Ibérica, Barcelona, Spain) were used. All experimental procedures were carried out in accordance with the guidelines of the European Communities Council Directive (86/609/EEC) and the Spanish Government (Real Decreto 1201/2005) and were approved by the local Animal Ethics Committee (Universidad de Navarra Institutional Committee on Care and Use of Laboratory Animals).

Experimental design

In the unilateral model (Figure 1A), baseline PET studies were performed one week before surgery. Sham and 6-OHDA-lesioned animals underwent PET studies at 1, 2, 3 and 6 weeks. The rotational screening by apomorphine administration was undertaken 3 weeks after the procedure. In the bilateral model (Figure 1B), PET studies were performed on sham and lesion animals 1, 3 and 6 weeks following the last 6-OHDA injection (see below). Behavior was analyzed longitudinally by measuring forelimb use and catalepsy-grid tests. All animals were sacrificed one week after last PET image acquisition and immunohistochemistry studies were carried out at post-mortem. Additionally, another 12 healthy rats underwent ^{11}C-DTBZ PET scanning and Magnetic Resonance Imaging (MRI) for the construction of a new ^{11}C-DTBZ PET template (Figure 2) to be used as reference for spatial normalization (*Collantes et al., 2009*). These 12 baseline studies were also used as baseline reference values for the bilateral model.

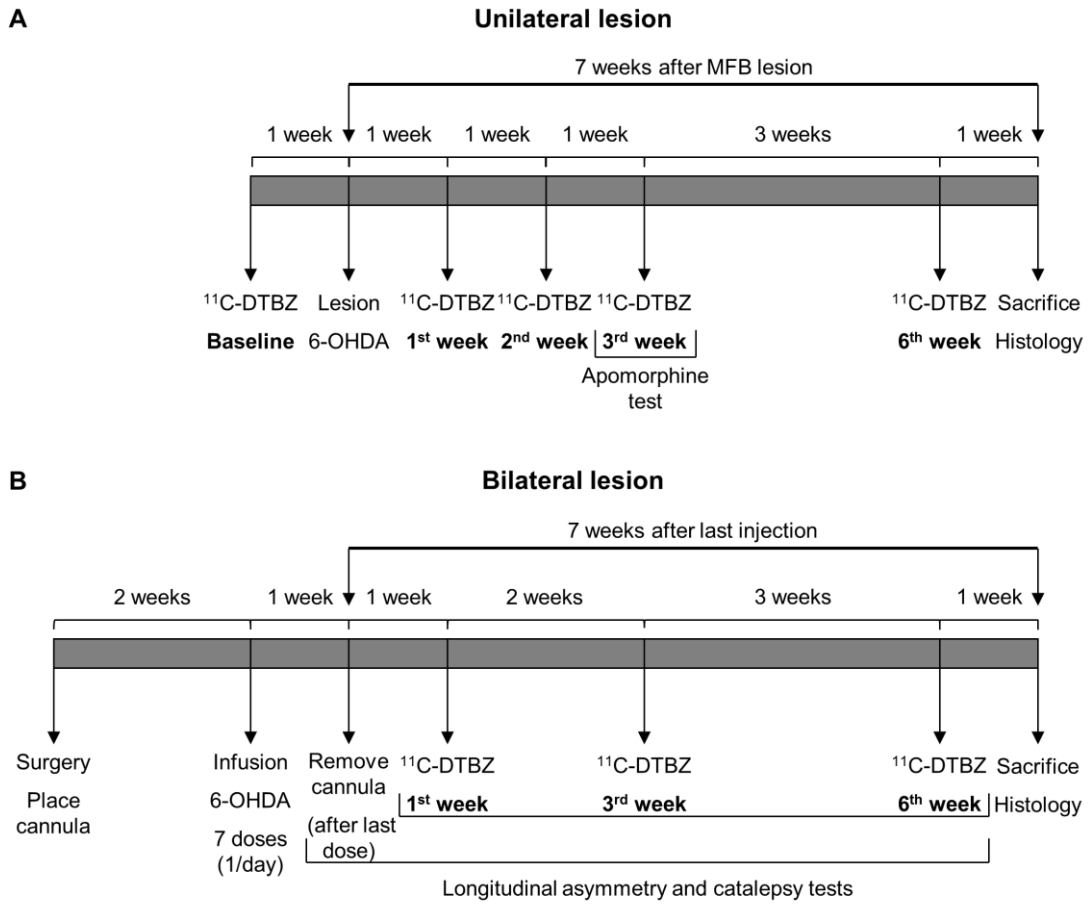

Figure 1: Experimental protocol design for unilaterally (A) and bilaterally (B) 6-OHDA-lesioned rat models of Parkinson's disease.

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Unilateral 6-OHDA lesion model

Twenty-two rats received a dose of 1 µg/µl in 4 µl of 6-OHDA (Sigma-Aldrich, St. Louis, MO, USA) dissolved in saline with 0.02 % ascorbic acid to induce the unilateral lesion. Sham animals (n=4) received 0.02% ascorbic acid diluted in saline. All surgical procedures were performed under xylazine (0.5 ml/kg, i.p.; Xilagesic®; Calier Laboratories) and ketamine (1 ml/kg, i.p.; Imalgene 1000®, Merial Laboratories) anesthesia. All rats were placed in a stereotaxic head frame with the incisor bar positioned 4.5 mm below the interaural line. The coordinates of the stereotaxic injection

were: 4.0 mm anterior to the interaural line, +1.3 mm lateral to the midline, 8.4 mm ventral to the surface of the skull. The neurotoxic was injected at a rate of 0.5 µl/min by an infusion pump into the left medial forebrain bundle (MFB).

Bilateral 6-OHDA lesion model

Rats (n=18) were anesthetized with xylazine and ketamine and placed in a stereotaxic frame with the incisor bar positioned 0 mm below the interaural line. A guide cannula was positioned in the third ventricle (0.8 mm posterior to Bregma, midline and 6.5 mm ventral below the dura). Dental cement was applied to fix it and the frontal bone of the skull was used as an anchor point. Two weeks after surgery, 100 µg of 6-OHDA diluted in 0.02 % ascorbic acid in a volume of 4 µl of saline on seven consecutive days (n=13) or vehicle (n=5) were injected daily. All solutions were administered with an injector cannula at a rate of 1 µl/min using an infusion pump. Rats were awake and freely moving during the infusion period.

Behavioral tests

The apomorphine-induced (0.05 mg/kg s.c.; dissolved in saline) rotational behavior of the animals in the unilateral model was measured 3 weeks after lesion (*Marin et al., 2009*; *Gago et al., 2013*). The rats were placed in circular cages and tethered to an automated rotometer linked to a computer (Panlab-Harvard Apparatus, Spain) to record for 60 min the number of ipsilateral and contralateral rotations. Rats with contralateral rotations were considered valid and included in the study (one rat was excluded only).

The evaluation of behavior in the bilateral model was performed by the catalepsy-grid (*Rodríguez et al., 2001*) and the forelimb use asymmetry (*Sutton et al.,*

2013) tests, which were carried out a few minutes before and 2 hours after each 6-OHDA or vehicle administration. After the last infusion the tests were performed once a day for 6 days and once per week up to 53 days. In the catalepsy-grid test, rats were gently placed on a wire grid at 45º above the surface. Catalepsy was considered to be present only when the animal remained completely immobile on the grid. We assessed the intensity of the cataleptic state by measuring the duration of immobile episodes during one minute, and the test was repeated four times, waiting 5 minutes between each observation period. The evaluation of the test was scored according with the following criteria: catalepsy present for 0 to 14 seconds, value of 0; from 15 to 29 seconds, value of 1; from 30 to 59 seconds, value of 2; and more than 60 seconds, the maxim value of 3. In addition, animals were placed in a transparent cylinder for 5 min to quantify the independent use of the right or left forelimb for contacting the wall (the limb use asymmetry test).

MicroPET scanning and MRI acquisition

Monoaminergic PET imaging was performed at Clínica Universidad de Navarra in a dedicated small animal Philips MOSAIC tomograph (Cleveland, OH, USA). Details of the methodology and the acquisition system have been published previously (*Huisman et al., 2007*).

All animals were anesthetized by isoflurane inhalation (2 % in 100 % oxygen gas) at the moment of the radiotracer injection and during PET image acquisition. Animals were placed on the PET scanner bed in prone position 15 min after radiotracer administration via the tail vein (37 ± 5 MBq in 0.2 ml of saline solution) to perform a static acquisition of 15 min. The radiotracer was synthesized as previously described (*Quincoces et al., 2008*), with minor modifications. Briefly, enantiomerically pure [^{11}C]-

(+)-α-dihydrotetrabenazine was obtained by O-methylation with [^{11}C]CH3I of the desmethyl precursor (+)-9-O-desmethyl-α-dihydrotetrabenazine (ABX, Germany) in basic medium (KOH in DMSO). The final compound was purified by solid phase extraction, solvents evaporated under He flow and dry residue dissolved in saline.

PET images were reconstructed using an iterative three-dimensional row action maximum likelihood algorithm (3-D RAMLA), with 2 iterations, on a 128×128×120 matrix, where the voxel size equals 1×1×1 mm. Corrections for decay, random coincidences and scattering were applied. Images were normalized for the injected radiotracer dose and animal body weight, resulting in semi-quantitative images representing the standardized uptake value (SUV).

MRI studies were performed at the Complutense University of Madrid and acquired in a BIOSPEC BMT 47/40 scanner (Bruker, Ettlingen, Germany) operating at 4.7 Tesla. Rats were anesthetized with the same mixture of isoflurane and oxygen. The acquisition parameters and protocols have been described previously (*Prieto et al., 2011*).

Analysis of ^{11}C-DTBZ PET images

Spatial normalization procedure for all PET images required the creation of a ^{11}C-DTBZ PET template (*Collantes et al., 2009*). The entire creation process is shown in Figure 2. Briefly, each PET image of 12 healthy animals was registered to the corresponding MRI scan. Then, co-registered PET images were spatially normalized applying the transformation matrix from each MRI scan normalization process, using a T2-weighted MRI rat brain template (*Schweinhardt et al., 2003*) as first reference. A provisional PET template was constructed as a smoothed average of these pre-normalized PET scans. Subsequently, all baseline PET studies underwent the process of

being normalized, averaged and smoothed in 3 iterations to finally obtain the ^{11}C-DTBZ PET template.

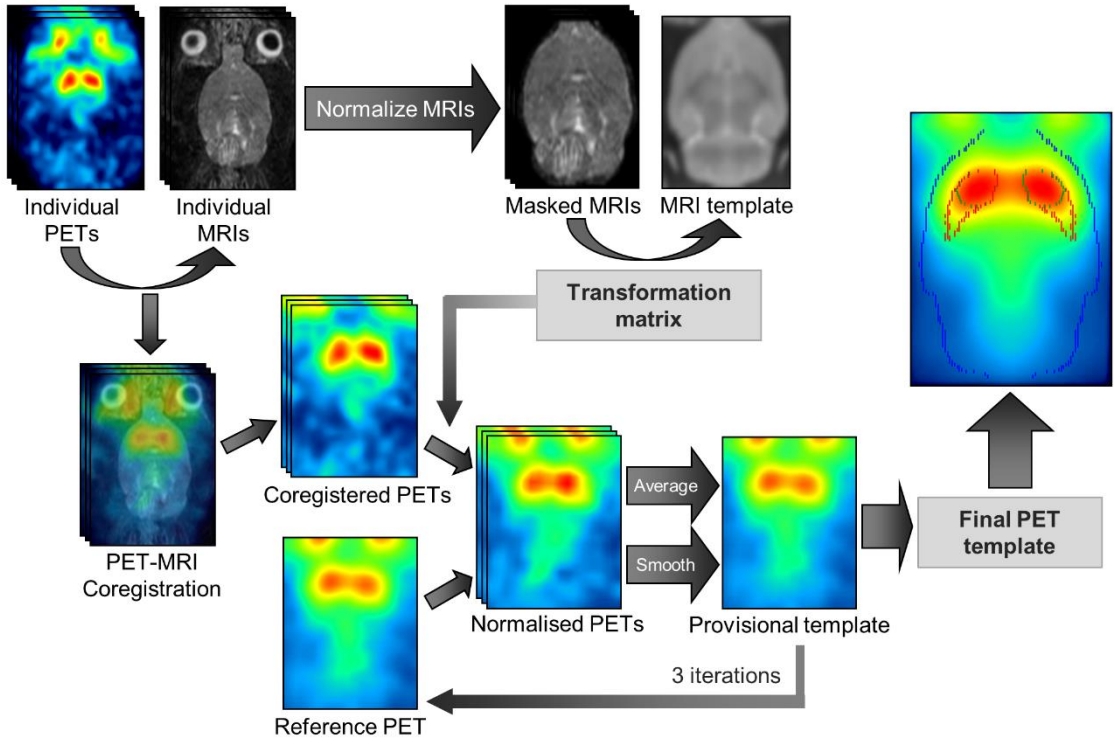

Figure 2: Step-wise process of the ^{11}C-DTBZ PET template of the rat brain from MRI and PET images at baseline state.

(double column fitting colored image)

All PET images were normalized into the standard stereotaxic space (*Paxinos and Watson, 2007*) using the ^{11}C-DTBZ PET template as reference with an automated algorithm based on mutual information using the PMOD fusion tool (version 3.2; PMOD Technologies Ltd., Adliswil, Switzerland). Final voxel dimensions were 0.2x0.2x0.2 mm after the interpolation step of the spatial normalization process.

Volume of interest (VOI) analysis was performed using PMOD software for the quantification of ^{11}C-DTBZ PET images. A brain atlas (*Frumberg et al., 2007*) that contains the delineation of the brain (2.073 cm^3) and striatum (0.044 cm^3) was used as guidance to manually draw reduced striatum (0.022 cm^3) and cerebellum (0.094cm^3)

spatially-standardized VOIs, which were used to quantify the average SUV value of these regions in the images (Figure 3).

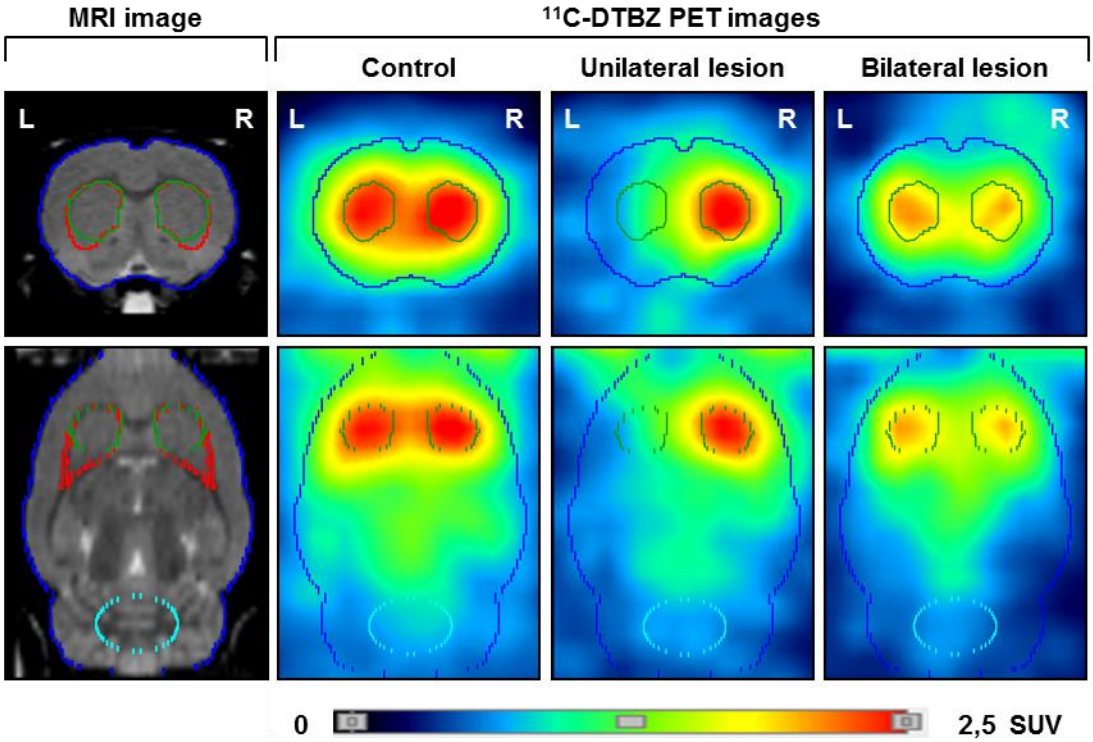

Figure 3: Representative coronal (top) and axial (bottom) sections of spatially normalized MRI scan and ^{11}C-DTBZ microPET images (at 1st week) of control, unilaterally- and bilaterally-lesioned animals. The figure shows the location of striatum (green) and cerebellum (light blue) anatomically-standardized volumes of interest (VOI) used to quantify standardized uptake value, brain outline (dark blue) and atlas-based striatum (red).

(double column fitting colored image)

Binding data were represented as striatal binding (SB) percentage measurements of a reference binding ratio (BR) value. BR was expressed as the average of the left or right striatal SUV value divided by the average of the cerebellar SUV value minus one; $BR = (SUV_{striatum} / SUV_{cerebellum}) - 1$. SB was estimated in the unilateral model as a percentage of the BR value in the lesioned side with respect to the intact BR value (2.10

± 0.41); $SB_{unilateral\ model}$ (%) = ($BR_{lesioned\ side}$ / $BR_{intact\ side}$) x 100. In the bilateral model, SB measurements represented the percentage of the BR value of each side with respect to the average BR value from both striatum of healthy animals (2.36 ± 0.24); $SB_{bilateral\ model}$ (%) = ($BR_{one\ side}$ / $BR_{baseline\ average}$) x 100.

Immunohistochemistry and optical density analysis

Immunohistochemistry studies were carried out for DAT detection using a standardized protocol in our laboratory (*Gago et al., 2013*). Coronal striatal sections (40 µm-thick) were obtained using a cryostat (Microm, Walldorf, Germany), thaw-mounted on Superfrost® Plus slides (Thermo Scientific), and stored at -80ºC until used. The sections were thawed, dried at room temperature and fixed in acetone for 10 min at 4ºC. They were immersed in 0.3% hydrogen peroxide in PBS for 10 min to block the endogenous peroxidase. At this point, the sections were incubated with rabbit serum with 0.1% Triton X-100 for 30 min followed by overnight incubation at 4 °C with goat anti-DAT monoclonal antibody (1:500 in PBS; sc-1433, Santa Cruz Biotechnology, Inc., Dallas, TX). Sections were subsequently incubated with biotinylated rabbit anti-goat Ig-G (Pierce, Thermo Fisher Scientific, Rockford, IL) for 30 min and an ABC Peroxidase staining kit (Vector Laboratories, Burlingame, CA) was used to carry out ABC staining. Finally, the sections were incubated with 3-3′-diaminobenzidine and 0.01% hydrogen peroxide for 10min. The slides were then washed with PBS, dehydrated in ascending alcohol concentrations, cleared in xylene and coverslipped in DPX mounting medium.

DAT immunolabeling was estimated by using Image J software (NIH, Bethesda, MD, USA), and the optical density of the striatal region was measured.

Statistical analysis

Statistical analyses were carried out using STATA software (StataCorp, Texas, USA). The difference in SB values between control groups (baseline and sham) and groups lesioned with the neurotoxin at different time points was set after testing the distribution of PET imaging data for normality (Shapiro-Wilk test) and homogeneity of variances (Levene's test). When normality could be assumed, data values were analyzed using two-way ANOVA (lesion group and repeated measures over time, factors); when not, a nonparametric Kruskal-Wallis equality-of-populations rank test was performed followed by Bonferroni correction and Mann-Whitney U-test (group factor).

A one-way ANOVA followed by Bonferroni test was used to assess differences in the number of apomorphine-induced rotations between groups and the results of forelimb use asymmetry tests were analyzed with non-parametric two-way repeated measures ANOVA and Bonferroni test. Significant changes in DAT immunolabeling between control groups and 6-OHDA lesioned animals were studied by performing a non-parametric one-way ANOVA followed by Dunn’s test for the unilateral model and Mann Whitney U-test for the bilateral model.

Correlation studies

The relationship between striatal dopaminergic activity as revealed by PET (week 6), histological and behavioral studies was evaluated by Pearson correlation.

Results

Figure 3 shows representative PET images at 1 week post-lesion. ^{11}C-DTBZ PET binding was reduced in the left striatum of unilaterally-lesioned animals. In the bilateral model a reduction of ^{11}C-DTBZ binding was found in the striatum bilaterally. No change was observed in control animals.

VOI based analysis of PET images

The changes in ^{11}C-DTBZ SB values in the unilateral and bilateral rat models associated with different degrees of 6-OHDA-induced lesion are summarized in Figure 4.

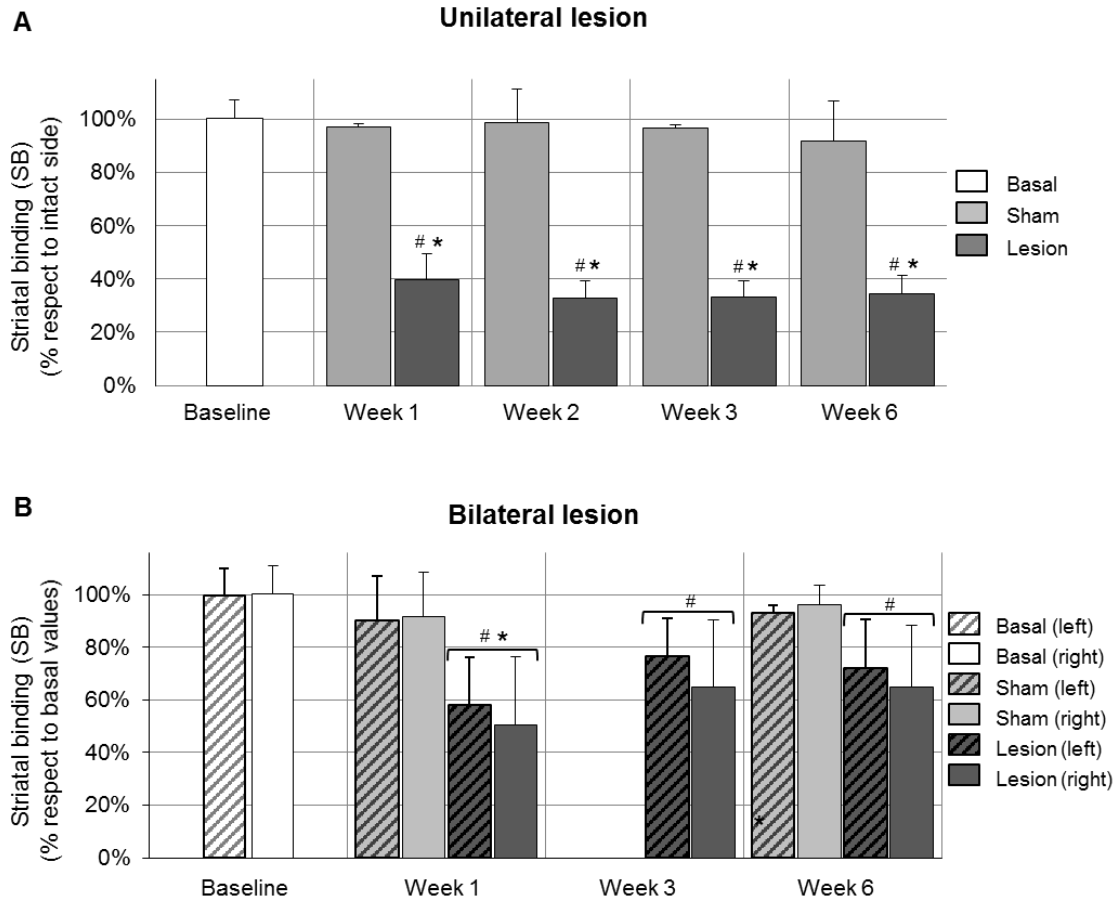

Figure 4: Time-course analysis of striatal binding (SB) values in the unilateral lesion (A) and the bilateral lesion (B) groups. SB data represent mean ± SD and are expressed as the percentage of the binding ratio (BR) values of the lesioned side with respect to the intact one in the unilateral model (A, sham n=4, lesioned n=22), and as the percentage of the BR values of each side with respect to the healthy average in the bilateral model (B, sham n=5, lesioned n=13). Parametric two way-ANOVA (A), and Kruskal-Wallis followed by Bonferroni correction (B); *p<0.001 sham vs lesion; $^{\#}$p<0.001 lesion vs baseline.

(double column fitting image)

In the unilateral model (Figure 4A), the lesion factor (6-OHDA effect) was statistically significant ($p<0.001$), and SB values were reduced at all time-points with respect to baseline values ($p<0.001$). At the first week post-lesion, animals exhibited a significant reduction of SB in the left striatum (60% of reduction). At the second week post-lesion, mean SB values decreased slightly (65% of reduction) and remained unchanged after 3 and 6 weeks post-lesion. The time factor and its interaction with the lesion factor were not significant ($p=0.746$), meaning that there were no changes over time for either sham or 6-OHDA lesion groups.

In the bilateral model (Figure 4B), no statistically significant differences between left and right hemisphere were found at any time point (Mann-Whitney U-test; $p=0.628$). Time of evolution after lesion did not reach significance ($p=0.089$), which could be related to large variability within groups. Thus, multiple comparisons (Kruskal-Wallis test) between all groups were performed assuming an adjusted p-value < 0.001 for significance by Bonferroni correction. At the first week, 6-OHDA bilateral lesion was associated with significant reduction (42% left, 49% right) of SB values with respect to baseline and sham groups. In the 3rd week after lesion, SB values showed an increase and the reduction was not significant in comparison with sham groups, but still was in comparison with baseline (24% left, 35% right). No further changes in ^{11}C-DTBZ SB occurred 6 weeks after the last 6-OHDA administration (28% left and 35% right reduction with respect to baseline).

Behavioral evaluations

In the unilateral model, sham animals showed no rotational asymmetry while 6-OHDA-lesioned rats exhibited increased contralateral turning under apomorphine with respect to the sham-treated group ($p<0.05$) (Figure 5A). In the forelimb use test (Figure 5B), bilaterally lesioned rats showed a significant reduction in the use of forelimbs with respect to baseline values ($p<0.001$) at all time-points; regarding the comparison with the sham group the difference was significant at days 11 and 18 after the last 6-OHDA injection. Unexpectedly, sham animals also showed reduced values respect to baseline (Figure 5B). The catalepsy score of lesioned animals was significantly different from baseline values ($p<0.001$) at all-time points analyzed, except for days 32 and 39 after the last injection of 6-OHDA (data not shown).

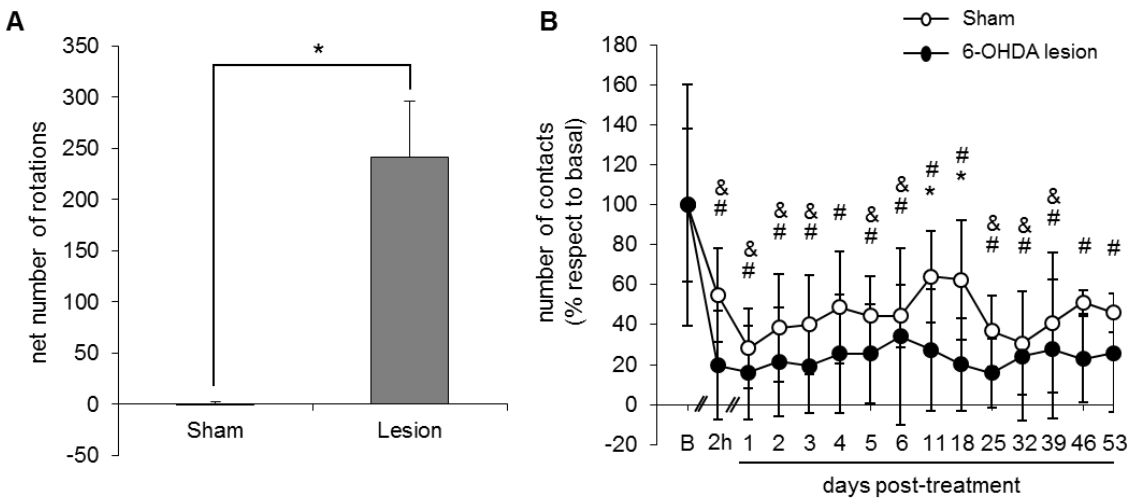

Figure 5. Evaluation of rotational behavior in animals with a unilateral lesion (apomorphine test, A, sham n=4, lesioned n=21) and the use of forelimbs (forelimb asymmetric use test, B, sham n=5, lesioned n=13) in the bilaterally lesioned rats (expressed as mean ± SD). Non-parametric one way-ANOVA (A) and two-way repeated measures ANOVA (B) followed by Dunn's and Bonferroni multiple comparison procedures, respectively. *p<0.05 sham vs lesion; $^{\#}$p<0.001 lesion vs baseline; $^{\&}$p<0.05 sham vs baseline.

(double column fitting image)

DAT immunoreactivity and striatal denervation

Optical density analysis in the unilaterally lesioned animals showed that 6-OHDA induced a significant DAT reduction in the left striatum (by 70%, $p<0.001$) (Figure 6A, B), which was associated to a reduction of the number of TH+ neurons in the SN by approximately 60% (*Tseng et al., 2005*). In the bilateral model, the reduction was of 28% (left) and 33% (right) with respect to sham levels ($p<0.001$) (Figure 6A, C) with no difference between striatal sides. This modest reduction is associated with ≈ 65% cell loss (TH+ stereology counting) in the SNpc bilaterally (data not shown; Quiroga et al In Preparation).

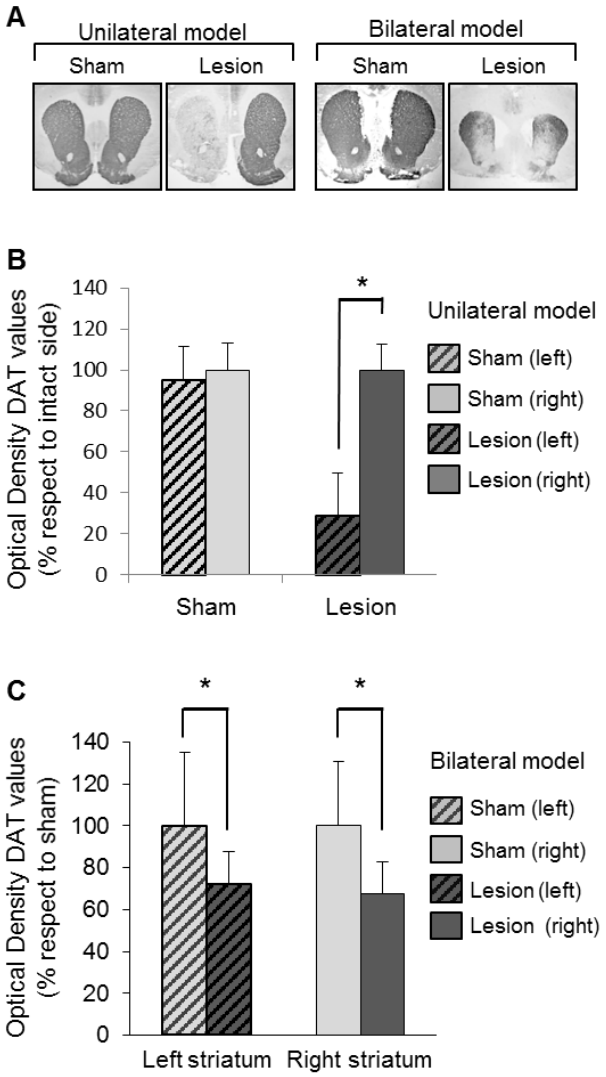

Figure 6. Representative photomicrographs showing dopamine transporter (DAT) immunoreactivity in the striatum (A). Semiquantitative optical density analysis of DAT immunolabeling in the striatum of unilateral (B) and bilateral (C) lesion models. Data represent mean ± SD and are expressed as a percentage respect to the intact side in the unilateral model (B, sham n=4, lesioned n=22) and as a percentage compared with sham animal values in the bilateral model (C, sham n=5, lesioned n=13). Non-parametric one-way ANOVA followed by Dunn's test and Mann Whitney U-test; *p<0.001 sham vs lesion.

(single column fitting image)

Correlations analysis

There was a highly significant correlation between striatal ^{11}C-DTBZ binding as measured *in-vivo* with microPET (week 6) and DAT striatal immunoreactivity *ex-vivo* values ($r = 0.95$, $p < 0.001$, Figure 7A). There was also a significant correlation between rotational values and PET studies at the third week after the unilateral lesions ($r = -0.91$, $p < 0.001$, Figure 7B). On the other hand, forelimb asymmetric use ($r = 0.27$, $p = 0.16$) and catalepsy-grid ($r = -0.28$, $p = 0.14$) scores were not significantly correlated with PET changes in the bilateral model.

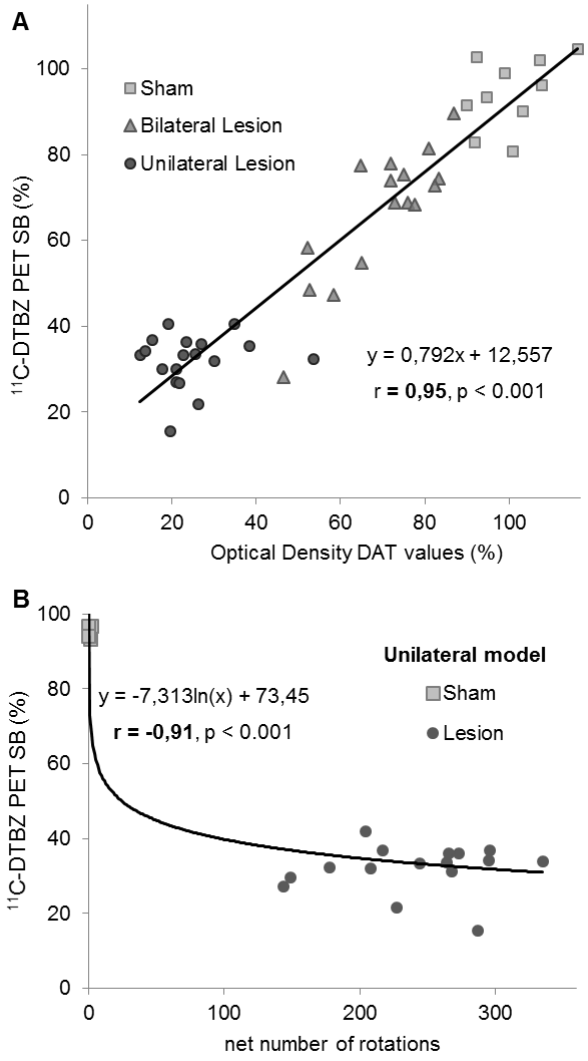

Figure 7. Pearson's correlation between PET values versus optical density DAT values (A) and rotational behavior values (B). There was a highly significant correlation between ^{11}C-

DTBZ striatal binding (SB) and DAT immunoreactivity labeling (r = 0.95, p < 0.001), and also with the rotational test in the unilateral model (r = -0.91, p < 0.001).

(single column fitting image)

Discussion

The present study describes the longitudinal evolution of ^{11}C-DTBZ binding in the striatum over 6 weeks in rats with a 6-OHDA-induced unilateral lesion in the medial forebrain bundle, and bilaterally lesioned rats after injection of the neurotoxin in the third ventricle for 7 consecutive days.

Imaging the nigro-striatal DA system in small animals has become now a feasible *in-vivo* monitoring method to ascertain the degree of dopaminergic depletion. Several studies (*Sossi et al., 2007; Collantes et al., 2008; Topping et al., 2010*) have indicated that changes in ^{11}C-DTBZ binding in the striatum can be directly related to reduction of VMAT2 density and the loss of dopaminergic terminals, suggesting that ^{11}C-DTBZ-PET may be a reliable index of nigro-striatal lesion. Indeed, our study shows that striatal dopaminergic depletion in two rat models can be ascertained and monitored *in-vivo* by ^{11}C-DTBZ PET with fairly good accuracy. We found an excellent correlation between DAT immunohistochemistry labeling in post-mortem tissue and ^{11}C-DTBZ PET (Figure 7A), and also with the rotational test in the unilateral model. This contrasts somehow with a previous study (*Strome et al., 2006*) using 6-OHDA-lesioned rats in the medial forebrain bundle, which described overestimation in the quantification of ^{11}C-DTBZ binding in large-sized lesions. These authors suggested that the inability to measure large asymmetries by PET in the unilateral model (comparing the lesioned side with respect to the intact side, using manually-defined VOIs) could be due to a partial volume effect as consequence of limited scanner resolution. Indeed, the limited spatial resolution of PET tomographs, particularly when studying small animals, and the

presence of low uptake areas make it difficult to delimit VOIs accurately. Consequently, the definition of VOIs is probably one of the most critical steps in the quantification procedure (*Sossi et al., 2007*) and introduces great intra- and inter-operator variability. In order to minimize such problems, we defined anatomically-standardized VOIs based and derived from a T2-weighted MRI (Figure 3) which closely fitted the ^{11}C-DTBZ binding pattern in the striatum. These reduced VOIs were placed into the common stereotaxic coordinate system (*Paxinos and Watson, 2007*) and provided a more accurate binding measurement than atlas-defined VOIs (*Sossi et al., 2007*). Moreover, we had created an ^{11}C-DTBZ PET template, meant to serve as a reference in spatial normalization procedures (*Collantes et al., 2009*) and quantify PET studies in an automated way with our anatomically-standardized VOI map. When images are spatially normalized and hence forced to match such a standardized space, those VOIs can be directly applied, reducing observer bias in VOI placement. Despite such improvements in the methodology, there is still a great difficulty to quantify ^{11}C-DTBZ PET in the bilateral 6-OHDA model, mainly as a consequence of the reference used in the process. As mentioned above, the reference used in the unilateral model is the intact side of the same rat, avoiding the inter-subject variability, whereas the average value of healthy rats was used as reference for all bilaterally-lesioned rats. This probably leads to larger variability in the comparisons. Indeed, we somewhat unexpectedly found that at 6 weeks ^{11}C-DTBZ PET values for bilaterally lesion rats were not statistically different from the sham-treated group. This contrasts with data for DAT striatal immunoreactivity that was significantly reduced in the lesion animals. We need to stress here that the differences in BP between lesion and sham groups at 6 weeks post-lesion was close to reach significance ($p=0.00646$), and when assuming an adjusted p-value < 0.001 for significance by Bonferroni correction, this was also close to threshold for statistical

significance. Thus, we believe that the variability implicit to PET assessment in the bilaterally depleted striatum rat mainly explains these findings. Another unexpected result was the finding of bilateral sham rats showing a significant reduction of forelimb use at several time points compared to their baseline levels. We think that habituation to the testing environment leading to reduced activity of the independent limb use upon repeated testing at short time intervals may well explain this observation. It could also occur that the reduced activity is related with a volume effect of the vehicle injected, but we found intact dopaminergic striatal terminals and no evidence of widespread brain damage.

Typically, a severe lesion of the nigro-striatal lesion is inflicted unilaterally in the 6-OHDA rat model, which precludes studying compensatory changes during the initial stage of dopaminergic loss (*Kirik et al., 1998; Rodríguez et al., 2001; Sadakierska-Chudy et al., 2010*). One original aspect of our study is the longitudinal follow-up of the PET changes over several weeks associated with two different administration modes of 6-OHDA. This allowed us to establish that the neurotoxin causes acute dopaminergic depletion that remained constant along time evolution in the unilateral model. In the novel bilateral-lesion rat model used here, which induced a slowly progressive lesion over several days of 6-OHDA administration, we found some relevant differences with respect to the classic 6-OHDA unilateral model. First, although the total 6-OHDA dose used was higher than in unilaterally lesioned animals, the bilateral lesions were not as severe as in the unilateral case. Secondly, and interestingly, striatal ^{11}C-DTBZ binding increased after the first week and striatal denervation (i.e. DAT immunostaining) was roughly half of SNpc cell loss, suggesting some degree of dopaminergic striatal recovery perhaps via sprouting of the remaining dopaminergic fibers. Some degree of motor improvement is well known to occur in the

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) monkey model (*Elsworth et al 2000*; *Mounayar et al., 2007; Blesa et al., 2012*) weeks/months after lesioning, but the intrinsic mechanisms underlying the recovery remain unraveled. We believe that the bilateral rat model may be applied to study compensatory mechanisms associated with different levels of nigro-striatal lesion as determined by ^{11}C-DTBZ PET.

Conclusions

In summary, we found a marked reduction in striatal ^{11}C-DTBZ-PET uptake in rats with 6-OHDA lesion, which was graded and proportional to the damage inflicted by the toxin. Dopaminergic depletion remained constant along time evolution in unilateral lesions whereas the bilateral model showed a modest increase in ^{11}C-DTBZ SB since the third week post-lesion. ^{11}C-DTBZ PET appears sensible to detect different degrees of changes of nigro-striatal innervation, particularly in the unilateral 6-OHDA model. SB values were highly correlated with histological and behavioral (for unilateral lesion) manifestations in 6-OHDA lesion rats. Accordingly, ^{11}C-DTBZ binding may be considered a useful tool to ascertain and define the degree of nigro-striatal lesion in rat models and putative changes occurring spontaneously, i.e. compensatory mechanisms, or induced by experimental therapies.

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