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RESEARCH****Research Report****In vivo type 1 cannabinoid receptor mapping in the 6-hydroxydopamine lesion rat model of Parkinson's disease**Cindy Casteels^{a,b,*}, Erwin Lauwers^{b,c}, Abdelbari Baitar^a, Guy Bormans^{b,d},
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ABSTRACT

Type 1 cannabinoid (CB1) receptors are expressed in high concentrations in the central nervous system, including the basal ganglia, and could have direct or indirect effects on motor behavior through modulation of dopaminergic, glutamatergic and GABA-ergic neurotransmission. Using the CB1 receptor radioligand [¹⁸F]MK-9470 and small-animal PET, we investigated for the first time in vivo cerebral changes in [¹⁸F]MK-9470 binding in the 6-hydroxydopamine (6-OHDA) rat model of Parkinson's disease (PD), parallel to dopamine transporter (DAT) imaging, tyrosine hydroxylase (TH) staining, and behavioral measurements. In the 6-OHDA model, relative [¹⁸F]MK-9470 PET binding decreased in the contralateral cerebellum (−9%, $p < 0.0004$) and caudate-putamen bilaterally (ipsilateral −8%, contralateral −7%; $p = 0.001$ and $p < 0.0003$, respectively). The number of TH⁺ neurons in the substantia nigra was inversely correlated to CB1 receptor binding in the ipsilateral cerebellum ($p = 1.10^{-6}$). The behavioral outcome was positively related to regional CB1 receptor binding in the contralateral somatosensory cortex ($p = 4.10^{-6}$). In vivo [¹⁸F]MK-9470 PET imaging points to changes in endocannabinoid transmission, specifically for CB1 receptors in the 6-OHDA model of PD, with mainly involvement of the caudate-putamen, but also distant regions of the motor circuitry, including the cerebellum and somatosensory cortex.

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1. Introduction

Type 1 cannabinoid (CB1) receptors are abundantly expressed in the basal ganglia (Herkenham et al., 1991), where they colocalize with D1 and D2 dopamine (DA) receptors in striatal neurons (Meschler and Howlett, 2001).

In the central nervous system, endogenous cannabinoid receptor agonists, such as anandamide (AEA) and 2-arachidonoylglycerol (2-AG) (Di Marzo and Deutsch, 1998), act as neuromodulators to fine-tune neuronal firing and neurotransmitter release in a dynamic, activity-dependent manner (Goutopoulos and Makriyannis, 2002). In particular, presyn-

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aptic modulation of DA-ergic, glutamatergic and GABA-ergic neurotransmission, the principal neurotransmitter systems in basal ganglia circuitry, has been described (Giuffrida et al., 1999; Beltramo et al., 2000).

The interdependency between CB1 and DA receptors and their high abundance in the striatal system has led to the hypothesis that targeting the CB1 receptor could be of value to improve motor deficits in neurodegenerative motor diseases such as Parkinson's disease (PD) (Di Marzo et al., 2004).

There is a growing body of ex vivo data suggesting striking alterations in CB1 receptor-mediated signaling in PD. Some of these alterations may reflect endogenous compensatory mechanisms as a result of the loss of DA-mediated control on basal ganglia activity, while others may contribute to the generation of the motor symptoms typically related to the parkinsonian state (Brotchie, 2003). Several studies have demonstrated that endocannabinoid transmission becomes overactive following DA denervation at intermediate and advanced stages (Fernandez-Ruiz et al., 2002; Brotchie, 2003; Fernandez-Ruiz, 2009). Accordingly, several parameters of CB1 receptor-related activity, such as the specific binding, mRNA expression and activation of GTP-binding proteins by agonists significantly increase in the basal ganglia after severe DA depletion both in humans (Lastres-Becker et al., 2001) and animals (Mailleux and Vanderhaeghen, 1993; Romero et al., 2000; Lastres-Becker et al., 2001). Other studies, however, have reported that CB1 receptors are normal in the striatum of 6-hydroxydopamine (6-OHDA)-lesioned rats with little to moderate nigral degeneration (Lastres-Becker et al., 2005) and of parkin-null mice at presymptomatic ages (Gonzalez et al., 2005). CB1 receptors are even down-regulated in reserpine-treated rats 18 hours after treatment (Silverdale et al., 2001) and in postmortem samples of patients with PD (Hurley et al., 2003). There is also evidence of a reduction in CB1 receptors at early and presymptomatic ages (≤ 12 months) in knockout mouse models of three genes linked to the development of PD (Garcia-Arencibia et al., 2009). Interestingly, at later symptomatic ages (> 18 months), the same up-regulatory responses of CB1 receptors were evident in these mice, suggesting CB1 receptor down-regulation or desensitization to be related to the early phase of the disease (Fernandez-Ruiz, 2009; Garcia-Arencibia et al., 2009).

The recent development of small-animal positron emission tomography (PET) scanners allows quantitative, non-invasive assessment of brain receptor binding in rodents. Currently, most radioligands for the CB1 receptor appear to be unsuitable for this purpose due to low brain uptake and high nonspecific binding (Gifford et al., 2002). Recently, we described the pharmacologic and preclinical characterization of a novel [^{18}F]-labeled CB1 receptor radioligand, [^{18}F]MK-9470 (Burns et al., 2007).

Given the growing body of predominantly ex vivo data suggesting therapeutic potential of the endocannabinoid system (ECS) in PD, we investigated for the first time voxel-wise in vivo changes in brain CB1 receptor binding in the 6-OHDA lesion rat model of PD. Furthermore, dopamine transporter (DAT) availability as measured using [^{18}F]-FECT, tyrosine hydroxylase (TH) activity and limb-use asymmetric behavior, all shown to be altered in PD (Antonini et al., 1996; Casteels et al., 2008), were quantified in the 6-OHDA animals

and within-group correlated to the regional CB1 receptor status.

The 6-OHDA rat model used in this study is a well-known acute model of PD produced by unilateral injection of the catecholamine-specific neurotoxin 6-OHDA in the substantia nigra (SN). This lesion causes a permanent DA-denervation in the ipsilateral caudate-putamen (Jeon et al., 1995) and produces unilateral hypokinesia and sensorimotor disintegration, overall mimicking several symptoms of human PD (Schwartz and Huston, 1996).

2. Results

2.1. Nigrostriatal 6-OHDA lesion

Quantitative analysis of TH-positive neurons in the pars compacta of the SN showed an average unilateral destruction of 98.1% (range 96%–99%) in the 6-OHDA rats. The nigral unilateral destruction was accompanied by reductions in DAT binding of the ipsilateral caudate-putamen. The affected-to-non-affected side binding index (BI) ratio, defined as DAT impairment, ranged from 0.9% to 17.5% after DA denervation. DA depleted rats also showed severe limb-use asymmetry during explorative behavior with on average only 2.14% ($\pm 2.67\%$) of the total number of wall contacts performed with the impaired forepaw. Individual behavioral, histological and [^{18}F]-FECT data are detailed in Table 1. Representative cross-sectional slices of the mean [^{18}F]-FECT BI of 6-OHDA-lesioned rats are presented in Fig. 1.

2.2. Categorical [^{18}F]MK-9470 analysis

Absolute [^{18}F]MK-9470 binding values were not significantly different between DA depleted rats and control animals. Average cross-sectional small-animal PET images of absolute [^{18}F]MK-9470 binding in controls and 6-OHDA-lesioned rats are shown in Fig. 1. As can be seen from this figure, [^{18}F]MK-9470 activity in the rat control brain shows a fairly homogenous

Table 1 – Percentage of intact dopaminergic neurons after unilateral infusion of 6-OHDA, as measured by caudate-putamen [^{18}F]-FECT binding in vivo using small-animal PET (DAT) and in vitro with TH-immunostaining (% TH), vs. the percent use of the impaired forelimb during the limb-use asymmetry test (%I).

No of animal	DAT impairment ^a	%I ^b	%TH ^c
1	2.9	5	3.4
2	6.0	0	1.2
3	17.5	0	n/a
4	3.1	0	3.0
5	2.3	5	1.1
6	3.4	0	1.8
7	0.9	5	0.89

n/a indicates not available.

^a Affected-to-non-affected side BI ratio of 6-OHDA.

^b % impaired paw contacts/20.

^c % number of TH⁺ neurons lesioned side/ number of TH⁺ neurons unlesioned side.

Fig. 1 – Mean $[^{18}\text{F}]$ MK-9470 and $[^{18}\text{F}]$ -FECT in 6-OHDA. Average cross-sectional small-animal PET images showing mean absolute $[^{18}\text{F}]$ MK-9470 binding (A) and mean $[^{18}\text{F}]$ -FECT binding index (B) of 6-OHDA-lesioned rats (right) in comparison to control data (left). Colored bars express SUV values for the CB1 receptor and binding indices for the dopamine transporter (DAT). The intersection points of the three planes have been set to the mid-striatal level (i.e., $(x,y,z)=(-2.9, -0.3, -5.6)$ Paxinos coordinates), which corresponds to the right hemisphere. Images are in neurologic convention.

and symmetrical uptake in the cortex and the highest relative activity in the cerebellum.

When looking at regional changes by relative scaling to the global mean tracer binding, $[^{18}\text{F}]$ MK-9470 values were decreased in the contralateral cerebellum (peak average value -9% , $p < 0.0004$ uncorrected), and in the caudate-putamen bilaterally (ipsilateral to lesion side: peak average value -8% , $p = 0.001$ uncorrected; contralateral: peak average value -7% , $p < 0.0003$ uncorrected) of 6-OHDA-lesioned rats, as seen in Fig. 2. Detailed cluster peak locations and p-values of the voxel-based categorical analysis using Statistical Parametric Mapping (SPM) are shown in Table 2.

2.3. Correlation analysis of $[^{18}\text{F}]$ MK-9470 with disease severity

As differences in disease severity measures (i.e., DAT impairment, %I and %TH; for a description see the method section) between both groups have previously been shown highly significant (Schwartz and Huston, 1996; Casteels et al., 2008), combining both groups for correlation analysis on these measures would only reflect group differences. We therefore only performed correlation analysis within the 6-OHDA group.

Correlation analysis revealed a positive relation between the behavioral outcome (%I) and relative $[^{18}\text{F}]$ MK-9470 binding in the contralateral secondary somatosensory cortex ($r = 0.99$; $p = 4.10 \times 10^{-6}$ uncorrected; Fig. 3A). The number of TH-positive neurons was inversely correlated to relative $[^{18}\text{F}]$ MK-9470 binding in the ipsilateral cerebellum ($r = -0.99$; $p = 1.10 \times 10^{-6}$

uncorrected, Fig. 3B). No homologous regional correlations of the degree of DAT impairment as determined by $[^{18}\text{F}]$ -FECT binding with relative $[^{18}\text{F}]$ MK-9470 uptake were observed. Cluster peak locations and p-values of the SPM correlation analysis are shown in Table 2.

3. Discussion

The ECS has been implicated in the symptomatology of PD, due to the marked changes in behavior produced by (endo) cannabinoids in patients with PD and in animal models (for review see Fernandez-Ruiz et al., 2002). Putative involvement of the ECS in DA neuron degeneration (Kim et al., 2005), as well as possible protective effects of (endo)cannabinoids in PD, have also recently been described (Lastres-Becker et al., 2005).

In this study, we have characterized the CB1 receptor binding in vivo in the brain of the 6-OHDA lesion rat model of PD for the first time, and found no overall group-based differences in absolute tracer binding. We however did detect small relative regional decreases in $[^{18}\text{F}]$ MK-9470 binding in the contralateral cerebellum and caudate-putamen bilaterally in comparison to controls.

As mentioned, previous reports relating changes in endo-cannabinoid signaling to PD, noted a time-dependent effect in activity with generally a hypoactive state at early phases and overactivity when the disease progresses (Fernandez-Ruiz, 2009). Specifically towards the 6-OHDA lesion model, the time-

Fig. 2 – Categorical comparison of [^{18}F]MK-9470 in 6-OHDA and controls. Coronal (top 4 rows) and axial (bottom 2 rows) brain sections showing significantly decreased relative CB1 receptor binding in the caudate-putamen bilaterally of 6-OHDA-lesioned rats ($n=10$; figure given at $p_{\text{height}} < 0.005$ uncorrected) compared to the controls ($n=10$). Significance is shown with a T statistic color scale, which corresponds to the level of significance at the voxel level. The distance between the sections is 1.00 mm with the position relative to Bregma on top of the sections in the left column. Images are in neurologic convention.

dependent effect has only been demonstrated for CB1 receptor mRNA levels of the ipsilateral caudate-putamen (Romero et al., 2000; Lastres-Becker et al., 2005), and in contrast to our findings not for CB1 receptor densities in this region. CB1 receptor densities of the ipsilateral caudate-putamen as measured using [^3H]CP55,940 autoradiography remained unaffected after moderate and severe DA-ergic injury at respectively 2, 7 and 17 weeks postlesioning (Romero et al., 2000; Gubellini et al., 2002; Lastres-Becker et al., 2005). Since in this study, an almost complete DA depletion resulted in modest changes 6 weeks after toxin injection, this might suggest that [^{18}F]MK-9470 PET is a more sensitive tool for the detection of CB1 receptor alterations.

A pattern of reduced CB1 receptor levels in the ipsilateral caudate-putamen of 6-OHDA rats is likely and fits well with the reported overactivity of the corticostriatal glutamatergic projections in this region, as seen in the same model at a similar time point (2–3 months) and following a comparable

loss of DA neurons in the SN pars compacta (>95%) (Gubellini et al., 2002). Indeed, within the caudate-putamen, besides few interneurons of the cholinergic population and some GABA-ergic ones that are labeled with parvalbumin (Hohmann and Herkenham, 2000; Fusco et al., 2004; Uchigashima et al., 2007), especially glutamatergic afferences coming from cortical structures contain CB1 receptors presynaptically (Kofalvi et al., 2005; Uchigashima et al., 2007). CB1 receptors exert a modulatory control on striatal glutamate release (Kofalvi et al., 2005). In line with this, CB1 receptor activation by HU-210 significantly reduced the frequency of spontaneous excitatory postsynaptic currents in corticostriatal 6-OHDA rat slices (Gubellini et al., 2002). Despite this agreement, however, other contributing mechanisms such as changes in receptor affinity or in conformational state can not be excluded.

This study shows that the unilateral 6-OHDA lesion also causes CB1 receptor-related abnormalities in the intact

Table 2 – Peak locations for the clusters in the group comparison and correlation analysis at $p_{\text{height}} \leq 0.001$ uncorrected.

Cluster level				Voxel level	Structure			Name	
p_{corr}	k_E	T	p_{uncorr}	Intensity Difference (%)	x	y	z		
Categorical analysis: [^{18}F]MK-9470									
1	0.39	827	4.50	<0.001	−5.2	3.8	0.4	8.0	Contralateral caudate-putamen
2	0.35	898	4.38	<0.001	−8.9	0.2	−13.6	−2.8	Contralateral cerebellum
3	0.29	983	4.17	<0.001	−7.2	1.2	−0.2	−3.8	Contralateral caudate-putamen
4	0.74	452	3.98	<0.001	−6.2	4.2	−10.4	−5.8	Contralateral cerebellum
5	0.62	569	3.57	0.001	−8.3	−1.6	−1.2	−3.8	Ipsilateral caudate-putamen
Correlation analysis: positive correlation with limb-use asymmetry test									
0.001	664	18.86	<0.001	−	5.6	−3.0	−4.2		Contralateral secondary somatosensory cortex
Correlation analysis: negative correlation with TH staining (remaining DA neurons)									
0.007	266	38.41	<0.001	−	−3.0	−12.6	−4.4		Ipsilateral cerebellum
P_{corr} at cluster level: the chance (p) of finding a cluster with this or a greater size (k_E), corrected for search volume.									
k_E = cluster extent.									
P_{uncorr} at voxel level: the chance (p) of finding (under the null hypothesis) a voxel with this or a greater height (T statistic), uncorrected for search volume.									
x = lateral distance in mm from the midline (negative values to the right side); y = anteroposterior location relative to Bregma (negative values: posterior to Bregma); z = dorsoventral position (based upon the Paxinos stereotactic atlas).									

(contralateral) caudate-putamen. This observation suggests the occurrence of adaptive changes in the plasticity of the basal ganglia circuitry. In line with our observations, bilateral decreases in striatal monoamine levels (i.e., DA, serotonin and metabolite content) have been reported following unilateral 6-OHDA injection in the SN (Pierucci et al., 2009). Also consistent with the CB1 receptor distribution in this region, chronic levodopa treatment increased striatal basal glutamate output on both sides in unilaterally lesioned 6-OHDA rats (Morgese et al., 2009). In addition, there is histologic evidence for interhemispheric projections from the SN to the striatum (Morgan and Huston, 1990), and electrophysiologic evidence

that activity in nigrostriatal DA-ergic cells (Castellano and Rodriguez Diaz, 1991), as well as in the subthalamic nucleus, are under control of the contralateral brain (Perier et al., 2000a, 2000b).

Selective DA-ergic lesions in the SN, caused by infusion of 6-OHDA, severely delay head movement reactions as a response to repetitive tactile stimulation (Schallert and Hall, 1988). Accordingly, we have also observed CB1 receptor changes in several brain areas involved in sensorimotor integration. The cerebellum, especially the dendritic tree of Purkinje cells, and the somatosensory cortex contribute in processing sensory input to guide motor acts (for review see

Fig. 3 – Correlation analysis of [^{18}F]MK-9470 with disease severity in 6-OHDA. (A) Positive correlation of relative [^{18}F]MK-9470 binding with impaired forelimb use (%I) in the 6-OHDA lesioned group (n=7). A significant cluster is seen in the contralateral secondary somatosensory cortex. (B) Negative correlation of relative [^{18}F]MK-9470 binding with the number of nigral TH-positive neurons in the 6-OHDA lesioned group (n=6). A significant cluster is seen in the ipsilateral cerebellum. The colored bars on the right express T score levels. The intersection points have been set to the maximal peak locations. Images are oriented in neurologic convention. Figs. given at $p_{\text{height}} < 0.005$.

Manzoni, 2007). The synthetic cannabinoid agonist WIN55,212-2 disrupts sensorimotor integration in rats, measured using a DA-regulated behavioral task (Schneider and Koch, 2002). Of special interest, intrastriatal nigral transplants restore sensorimotor deficits in rats with unilateral 6-OHDA lesions of the medial forebrain bundle (Nikkhah et al., 1993). In line with our findings, the magnitude of improvement is dependent on the DA levels of the caudate-putamen. Also, in symptomatic PD patients and asymptomatic *Parkin* mutation carriers, abnormal sensorimotor connectivity has been reported in transcranial magnetic stimulation studies (Lewis and Byblow, 2002; Baumer et al., 2007), although no relation with disease severity and consequently striatal DA content was found there. It would be interesting to further evaluate whether these correlations also remain visible in a more heterogeneous group, i.e., in 6-OHDA rats with continuous mild to severe DA-ergic lesions, or to evaluate whether these correlations are absent in control animals, to further strengthen their involvement in disease severity.

The limb-use asymmetry test and TH-immunostaining were performed, as mentioned in the experimental procedures, 19–37 weeks following the administration of 6-OHDA intranigally, which is a time interval of 13–31 weeks from the [^{18}F]MK-9470 PET imaging. Putative alterations of the DA-ergic lesions occurring within that time interval are unlikely to have affected the behavioral and histologic outcome. It has clearly been shown that lesions induced by 6-OHDA result in stable motor deficits over time (range 3–19 weeks) when the cell loss is greater than 70% (Iancu et al., 2005). Of all behavioral tests evaluated by Iancu and coworkers, i.e., various drug-induced and spontaneous motor tests, the limb-use asymmetry test was one of the most sensitive and reliable ones in detecting loss of TH-immunoreactive cells in the SN. Although DAT imaging enables the measurement of continuous mild to severe DA-ergic lesions and has a more robust quantitative outcome compared to the limb-use asymmetry test, it is also more sensitive to compensatory mechanisms (Sossi et al., 2009), which probably explains the absence of correlations between DAT impairment and [^{18}F]MK-9470 activity.

In the design of this study, we have explicitly set the aim to investigate CB1 receptor network alterations by using both absolute and relative [^{18}F]MK-9470 measurements. It is known that there is a higher physiologic interindividual variability of absolute regional [^{18}F]MK-9470 determinations in the human brain, in the order of several tens of percents (Barrero et al., 2005; Burns et al., 2007). Relative measurements have the advantage of being much more sensitive, allowing changes of 5%–10% to be measured using Statistical Parametric Mapping (Van Laere et al., 2002), as proven here. The use of female rats in this study, of which is known that the estrous cycle influences CB1 receptor expression in certain rat brain regions (Rodriguez De Fonseca et al., 1994), is only a minor potential bias, which may have increased the intersubject variability of our [^{18}F]MK-9470 measurements, but does not question the current findings. Another limitation, however, might be the use of controls animals (without surgery) as the basis for comparison, although the adequateness of sham-infused rats for investigating the ECS has also been questioned. Comparing unilateral intranigral 6-OHDA injection in rats to sham

infusion and no surgery, Fernandez-Espejo et al., revealed effects on NAT (N-acyl-transferase) and FAAH (fatty acid amidohydrolase) activity specific for the sham group (Fernandez-Espejo et al., 2004). Effects were suggested to be caused by a long-term inflammatory effect of saline and 0.2% ascorbic acid injection. Since both NAT (synthesizing enzyme) and FAAH (degradative enzyme) regulate the amount of AEA in the brain, possible side effects of sham lesions on CB1 receptor levels are also not unlikely. Besides, most alterations in CB1 receptor binding observed in our study were seen after group-comparison of the contralateral hemispheres, which are both unaffected by needle insertion.

Although 6-OHDA neurotoxicity provokes DA-ergic molecular alterations comparable to those seen in PD, the pathologic hallmark of human sporadic PD, Lewy body formation, has not been convincingly demonstrated in this rat model (Blum et al., 2001). Furthermore, the acute nature and large intensity of the 6-OHDA lesion is at odds with the human condition in regard to time progression and severity. These findings, together with the suggestion that major species specific differences may exist in the molecular endocannabinoid changes triggered by DA denervation (Maccarrone et al., 2007), reinforce the importance of translational research. Furthermore, the fact that endocannabinoid changes differ at different time points during the progression of parkinsonism (Fernandez-Ruiz, 2009), supports the importance of longitudinal study designs. Therefore, careful comparison to the human condition, and to other time points following 6-OHDA toxicity, are needed to further evaluate the functional significance of our current findings.

In conclusion, this study suggests *in vivo* changes in endocannabinoid transmission specific for CB1 receptors in the 6-OHDA brain lesion rat model of PD. Voxel-based analysis points to involvement of the caudate-putamen, but also to distant regions of the motor circuitry, including the cerebellum and somatosensory cortex. Translational *in vivo* studies in humans using the same imaging technique are needed to demonstrate whether CB1 receptor imaging may provide direct proof-of-principle for possible ECS-related changes in the brain of PD patients and effects of drug interventions.

4. Experimental procedures

4.1. Animals

Experiments were conducted on 20 female Wistar rats (body weight range at the start of the experiment 230–284g). All animals were housed 3 to a cage, at an average room temperature of 22 °C and a 12-h light/dark cycle. Food and water were given *ad libitum*. The research protocol was approved by the local Animal Ethics Committee and was according to European Ethics Committee guidelines (decree 86/609/EEC).

4.2. Nigrostriatal 6-OHDA lesion

All surgical procedures were performed under ketamine (60 mg/kg intraperitoneal (i.p.)) and medetomidine (0.4 mg/kg i.p.) anesthesia using aseptic procedures. The rats assigned to the

6-OHDA-lesioned group ($n=10$) were placed in a stereotactic head frame (Stoelting, Wood Dale, Illinois) and a small hole was drilled in the skull in the appropriate location using Bregma as reference. The neurotoxic lesions in the right SN were made by injecting 24 μg 6-OHDA (Sigma, St. Quentin-Fallavier, France) at the following coordinates: antero-posterior (AP) -4.8 mm, lateral (L) 2.1 mm and dorsoventral (DV) 7.2 mm. 6-OHDA was dissolved in 3 μl of 0.9% sterile NaCl containing 0.1% ascorbic acid. After the injection, the needle was left in place for an additional 10 min before being slowly withdrawn from the brain. No surgery was performed on the control animals ($n=10$).

4.3. Radiotracer synthesis

CB1 receptor imaging was performed in all animals using the radioligand [^{18}F]MK-9470 (N-[2-(3-cyano-phenyl)-3-(4-(2-[^{18}F] fluoroethoxy)phenyl)-1-methylpropyl]-2-(5-methyl-2-pyridyloxy)-2-methylpropanamide), which is characterized by high specificity and high affinity for the CB1 receptor (rat IC_{50} 0.9 nM) (Burns et al., 2007). The precursor for tracer synthesis was obtained from Merck Research Labs (West Point, PA, USA) and labeling was performed using a [^{18}F]-ethylbromide procedure as described previously (Burns et al., 2007). The final product was obtained after high performance liquid chromatography (HPLC) separation and had a radiochemical purity >95%. Specific activity was always higher than 10 GBq/ μmol .

Functional images of the presynaptic DA system were obtained in 6-OHDA rats only ($n=8$) using the DAT radioligand [^{18}F]-FECT (2'-[^{18}F]fluoroethyl-(1R-2-exo-3-ene)-8-methyl-3-(4-chlorophenyl)-8-azabicyclo[3.2.1]octane-2-carboxylate (Wilson et al., 1996) [^{18}F]-FECT binds selectively to DAT with high affinity ($K_i=9$ nM with respect to PE2I (Chitneni et al., 2008)). The synthesis of [^{18}F]-FECT PET radiotracer was performed according to the procedure by Wilson et al., but using 2-[^{18}F]-fluoroethyltrifluoromethanesulfonate instead of 2-[^{18}F]-fluoroethylbromide (Wilson et al., 1996).

4.4. Data acquisition and reconstruction

Small-animal PET imaging was performed using a LSO detector-based FOCUS 220 tomograph (Siemens/Concorde Microsystems, Knoxville, TN), which has a transaxial resolution of 1.35 mm full-width at half-maximum. Data were acquired in a $128 \times 128 \times 95$ matrix with a pixel width of 0.475 mm and a slice thickness of 0.796 mm. The coincidence window width was set at 6 ns. Prior to small-animal PET imaging, rats were anesthetized with an i.p. injection of 50 mg/kg sodium pentobarbital (Nembutal, Ceva Sante Animale, Brussels, Belgium). Approximately 18 MBq (500 μCi) of each radioligand (specific activity range 53–541 GBq/ μmol ; injection volume 500 μl) was injected into the tail vein using an infusion needle set.

Imaging studies of the 6-OHDA-lesioned rats were performed on average 5.8 (1.9 SD) and 9.2 (1.7 SD) weeks postlesioning for [^{18}F]MK-9470 and [^{18}F]-FECT, respectively. Sixty-minute dynamic [^{18}F]MK-9470 acquisitions were performed after overnight fasting and were started immediately after tracer injection. [^{18}F]-FECT measurements were obtained

during 40 min, starting 3 h postinjection. The acquisition timing rationale and kinetics of both [^{18}F]MK-9470 and [^{18}F]-FECT in rats have been described previously (Casteels et al., 2006; Goffin et al., 2008).

For quantification purposes, sinograms were reconstructed using filtered backprojection (FBP). No corrections were made for attenuation or scatter.

4.5. Behavioral testing

After small-animal PET imaging, the limb-use asymmetry test, a modified version of the cylinder test for rats (Schallert and Tillerson, 2000) was performed on the 6-OHDA-lesioned rats ($n=7$; 24 weeks postlesioning; range 19–33 weeks) to assess gait disturbances. Forelimb use during explorative activity was analyzed by videotaping rats in a transparent glass cylinder (20 cm diameter and 38 cm height). A mirror was placed behind the cylinder at an angle to enable the rater to record forelimb movements when the animal was turned away from the camera. The cylindrical shape encourages vertical exploration of the walls. No habituation to the cylinder prior to videotaping was allowed. The test was performed between 09.00 h and 18.00 h after overnight fasting to increase behavioral activity. To stimulate rats that showed little or no tendency to explore, the following methods were used in this given order: (i) turning the lights in the room down for a few seconds; (ii) taking the rat out of the cylinder and cleaning the cylindrical environment before putting the animal back (Lundblad et al., 2002). All scoring was done using computerized digital videorecordings with slow motion.

The number of wall contacts performed independently with the left and right forepaw were counted up to a total number of 20 wall contacts per rat and session. The number of 'both limb' placements (i.e., simultaneous) were excluded. Only supporting contacts were counted, i.e., full appositions of the paw with open digits to the cylinder wall. Data were expressed as percent use of the impaired forelimb relative to the total number of wall contacts (%I).

4.6. Immunohistochemistry

At the completion of the in vivo experiments, the remaining DA-ergic neurons in the SN pars compacta of the 6-OHDA rats ($n=6$) were visualized by in vitro tyrosine hydroxylase staining. Rats were sacrificed by sodium pentobarbital overdose and perfused transcardially with 300 ml phosphate buffered saline (PBS), followed by 300 ml of 4% paraformaldehyde at a flow rate of 30 mL/min before decapitation. Their brains were carefully removed from the skull and postfixed in 4% paraformaldehyde solution overnight. Coronal brain sections measuring 50 μm in thickness were cut with a vibratome (Leica, Microsystems, Wetzlar, Germany), collected free floating and stored at 4 °C in PBS buffer containing 0.1% sodium azide. Every fifth section of the SN was then treated with 3% hydrogen peroxide (H_2O_2) to block endogenous peroxidase activity and incubated overnight with primary rabbit anti-TH antibody (Chemicon, Temecula, CA; dilution of 1:5000) in 10% normal swine serum. After three rinses with PBS-0.1% Triton X-100, sections were incubated with biotinylated swine antirabbit secondary antibody for 30 min at room

temperature, followed by incubation with Strept-ABC-HRP complex (DAKO, Glostrup, Denmark). Before visualization of the TH-immunoreactive neurons with 3,3-diaminobenzidine (DAB), H₂O₂ was added to run the reaction to completion. Sections were mounted on gelatinized slides, dehydrated in increasing ethanol concentrations and cover slipped with mounting fluid.

The number of TH-immunopositive neurons in the SN pars compacta was counted using an unbiased stereologic method: the optical fractionator (StereoInvestigator, MicroBrightField Inc, Colchester, VT, USA). Quantification was performed on average 29 weeks postlesioning (range 24–37 weeks) with a total of 7 to 9 sections per animal. The remaining DA-ergic cells on the lesioned side were expressed as a percentage of the cell numbers on the contralateral (unlesioned) side (%TH).

4.7. Small-animal PET data processing and Statistical Parametric Mapping

Parametric images based on standardized uptake values (SUV) (activity concentration (MBq/ml) × body mass(g)/injected dose (MBq)) were generated as a measure of absolute CB1 receptor binding (Burns et al., 2007). No significant differences in weight or injected activity were present between 6-OHDA and control rats (data not shown). We investigated absolute as well as relative uptake. Relative [¹⁸F]MK-9470 uptake was expressed as SUV values normalized on whole-brain SUV. Parametric DAT binding index (BI) images were constructed by reference to the cerebellum: (tissue/average activity concentration cerebellum)–1.

To obtain maximal use of image information, images were analyzed on a voxel-by-voxel basis using SPM2 (Statistical Parametric Mapping, Wellcome Department of Cognitive Neurology, London, United Kingdom; <http://www.fil.ion.ucl.ac.uk/spm/>). The procedure of spatial normalization and its validation have been described previously (Casteels et al., 2006). This methodology allows reporting results in coordinates directly corresponding to the Rat Brain Paxinos coordinate system. Briefly, SPM analysis was carried out using a categorical subject design (conditions: disease vs. controls) on parametric [¹⁸F]MK-9470 images of 6-OHDA-lesioned rats. Spatially normalized images were masked to remove extra-cerebral signal that would disrupt the global normalization. All images were smoothed with an isotropic Gaussian kernel of 1.6 mm. For analysis of absolute receptor binding, no proportional scaling was used and an analysis threshold of 0.8 of the mean image intensity was applied. To exclude false-positive findings, T-map data were interrogated at a peak level of $p \leq 0.001$ (uncorrected) and extent threshold $k_F > 200$ voxels (1.6 mm³), as described previously (Van Kuyck et al., 2007). Only those clusters that were significant at the $p < 0.05$ (corrected) level were considered, unless clusters were located in regions of a priori knowledge. For analysis of relative [¹⁸F]MK-9470 uptake, proportional scaling was used.

In addition, a within-group voxel-based correlation analysis between relative [¹⁸F]MK-9470 uptake and disease severity measurements was performed using the following covariates: [¹⁸F]-FECT binding to characterize DAT impairment, %I and %TH. DAT values were determined by a

predefined volume-of-interest (VOI) analysis based on striatal volume definition on the Paxinos atlas (Casteels et al., 2006). For DAT impairment, the affected-to-non-affected side BI ratio was obtained.

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