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Exploration of the Dopamine Transporter: *In Vitro* and *In Vivo* Characterization of a High-Affinity and High-Specificity Iodinated Tropane Derivative (*E*)-*N*-(3-iodoprop-2-enyl)-2β-carbomethoxy-3β-(4′-methylphenyl)nortropane (PE2I)

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ABSTRACT. For the diagnosis and follow-up of neurodegenerative diseases, many cocaine derivatives have been proposed as radioligands to explore the dopamine transporter. As none of them have all the criteria of specificity and kinetics for human use, we have developed a new derivative, (*E*)-*N*-(3-iodoprop-2-enyl)-2β-carbomethoxy-3β-(4′-methylphenyl)nortropane (PE2I), which displays promising properties. We report the characterization of PE2I *in vitro* on rat striatal membranes and *in vivo* in rats and in monkeys. PE2I had a high affinity (Kd = 0.09 ± 0.01 nM) and high specificity for the dopamine transporter. In rats we observed a high accumulation in the striatum; by contrast, a very low fixation was measured in the cortex. Moreover, a preinjection of a saturating dose of GBR 12909 prevented the striatal accumulation of PE2I by 74%. These results confirmed the specificity of PE2I for the dopamine transporter. *In vivo* in monkeys, SPECT studies showed a high accumulation in striatum. Moreover, an equilibrium state was obtained 1 h after injection. PE2I seemed to be the most promising ligand for the dopamine transporter exploration by SPECT using a single-day protocol. NUCL MED BIOL 25;4:331–337, 1998. **© 1998 Elsevier Science Inc.**

KEY WORDS. Dopamine transporter, Cocaine, SPECT, Tropane

INTRODUCTION

Several neurodegenerative diseases such as Parkinson's disease are characterized by degeneration of the dopaminergic neurons resulting in a loss of dopaminergic transporters localized on the nerve endings in striatum. Nuclear medicine may play an important role in the diagnosis and follow-up of this disease; indeed, conventional anatomical exploration fails to detect any change in brain in this situation. In Parkinson's disease, radiopharmaceuticals that bind to dopamine D2 receptors localized on the postsynaptic side may provide some information (24), but the most important change is observed on the presynaptic side. It has been demonstrated by both *in vitro* postmortem studies (17, 21) and by PET (12) that there is a decrease in dopamine transporter sites during Parkinson's disease. It can therefore be assumed that *in vivo* imaging of this transporter by SPECT exploration would be very useful for the diagnosis and evaluation of treatments. Cocaine is well-known for its ability to inhibit dopamine uptake by acting directly on the presynaptic dopamine transporter. Many iodinated cocaine derivatives have therefore been synthesized for SPECT, the most widely used being βCIT, and promising results have already been obtained with [^{123}I]βCIT in humans (1, 15, 25).

However, βCIT has two major problems. First, this ligand is not specific for the dopamine transporter; it also has a great affinity for the serotonin transporter (2). Second, its kinetics are not very appropriate to human scintigraphy with 123-iodine. Indeed, the equilibrium state necessary to perform quantification is obtained only 24 h postinjection (7, 20). Many other derivatives have therefore been proposed to improve specificity and to obtain accurate kinetics, including FE-βCIT (18), FP-βCIT (5, 16, 23), IPT (14, 19, 22), and βCDIT (9). Because none of these derivatives display optimum kinetics and specificity properties, we have developed a structure-activity relationship with two families of tropane where nitrogen and phenyl substitutions were performed in order to select the best ligand for SPECT application. From this study we have demonstrated that an iodopropenyl group link at the nitrogen bridgehead, associated with a 4′-methylphenyl link at C-3, provides the most promising tracer of these series (10). We then selected (*E*)-*N*-(3-iodoprop-2-enyl)-2β-carbomethoxy-3β-(4′-methylphenyl)nortropane (PE2I) for further biological characterization. Thus, the aim of the present work was to characterize PE2I *in vitro* and *in vivo*. *In vitro* binding studies were evaluated on rat striatal membranes, and *in vivo* experiments were performed with biodistribution and autoradiographic studies in rats and with SPECT imaging in monkeys.

MATERIALS AND METHODS

Radiolabelling Procedure

Both [^{125}I] and [^{123}I](*E*)-*N*-(3-iodoprop-2-enyl)-2β-carbomethoxy-3β-(4′-methylphenyl)nortropane (PE2I) were prepared by iododestannylation of the tributyltin precursor (Fig. 1). To a vial contain-

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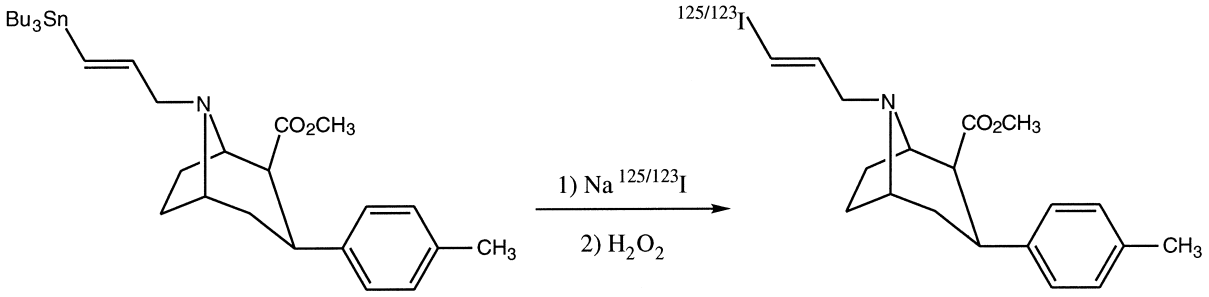

FIG. 1. Preparation of [$^{125/123}$I]PE2I.

ing 50 μg of the stannyl precursor were added 50 μL EtOH, 50 μL HCl (0.1 N), 37 MBq [^{125}I]NaI (in 10 μL NaOH 0.1 N, specific activity: 75 TBq/mmol; Amersham, UK) or 74 MBq [^{123}I]NaI (in 100 μL NaOH 0.1 N, specific activity >185 TBq/mmol; Cis Bio, France) and 50 μL of 3% w/v hydrogen peroxide. The reaction was allowed to stand at room temperature for 15 to 30 min, quenched with 100 μL $Na_2S_2O_5$ (300 mg/mL), basified with saturated $NaHCO_3$ and extracted with ethyl acetate (3 × 1 mL). The combined ethyl acetate extracts were evaporated under a nitrogen stream, and the residue was dissolved in 200 μL of the HPLC mobile phase. The radioiodinated solution was purified by HPLC using a reverse-phase column C-18 and a mixture of MeOH/H_2O/Et_3N: 75/25/0.2 as mobile phase (flow rate = 1 mL/min). The fraction eluted at the retention time of PE2I was collected and passed through a SEP-Pak C18 column. The radioiodinated product was then eluted with 2 × 1 mL EtOH and evaporated under a nitrogen stream. Structural identification and radiochemical purity were checked by HPLC using cold PE2I as reference under the same conditions.

In Vitro *Binding Studies*

TISSUE PREPARATION. Experiments in rats were carried out in compliance with appropriate guidelines from the Ministère de l'Agriculture, France. Male Wistar rats weighing 250 g were used. They were rapidly decapitated and striata were dissected on ice. Tissue was prepared according to Bonnet and Costentin (4). The tissue was homogenized in 10 vol of 0.32 M sucrose using Ultraturrax (Ultraturrax T25). After 1000 *g* centrifugation for 10 min at 2°C, the supernatant and the pellet were collected separately. The pellet was homogenized, washed, and centrifuged as described above. Supernatants were pooled and centrifuged at 17,500 *g* for 30 min at 2°C. Pellets were homogenized in 20 vol of the assay buffer and then centrifuged at 50,000 *g* for 10 min at 2°C. The pellets were suspended in the minimum volume of assay buffer, and the protein concentration was determined according to Bradford (6) using BSA as standard.

[^{125}I]PE2I BINDING ASSAY. The binding studies of PE2I were conducted on rat striatal membranes by saturation assays, and the pharmacological characterization was determined by competition with drugs known to bind to the DA, 5HT, and NE transporters.

GBR 12909 and nisoxetine were purchased from RBI Bioblock (Ilkirch, France); cocaine was purchased from Cooper (France), and paroxetine was a gift from Beecham (France). Preparation of the membrane proteins was conducted in the incubation buffer (Tris HCl 50 mM; NaCl 120 mM; KCl 5 mM, pH 7.4) as previously described.

For saturation studies, different concentrations of [^{125}I]PE2I (from 0.01 nM to 1.5 nM) were incubated with 30 μg of proteins in a total volume of 0.2 mL. Nonspecific binding was determined using 30 μM cocaine as ligand. Samples were incubated at 37°C for 1.5 h

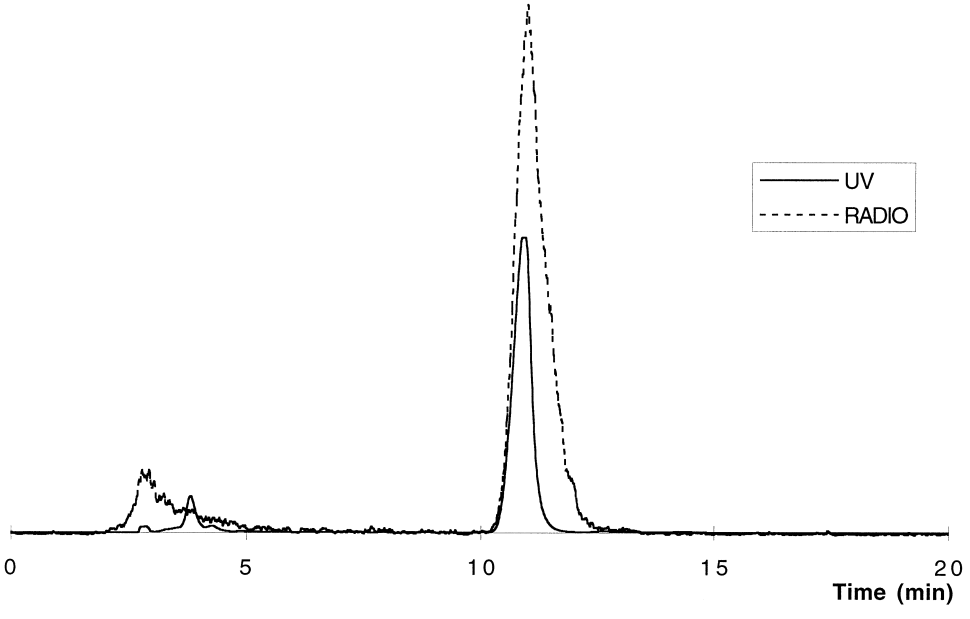

FIG. 2. Co-injection of cold PE2I and [^{125}I]PE2I on the HPLC system.

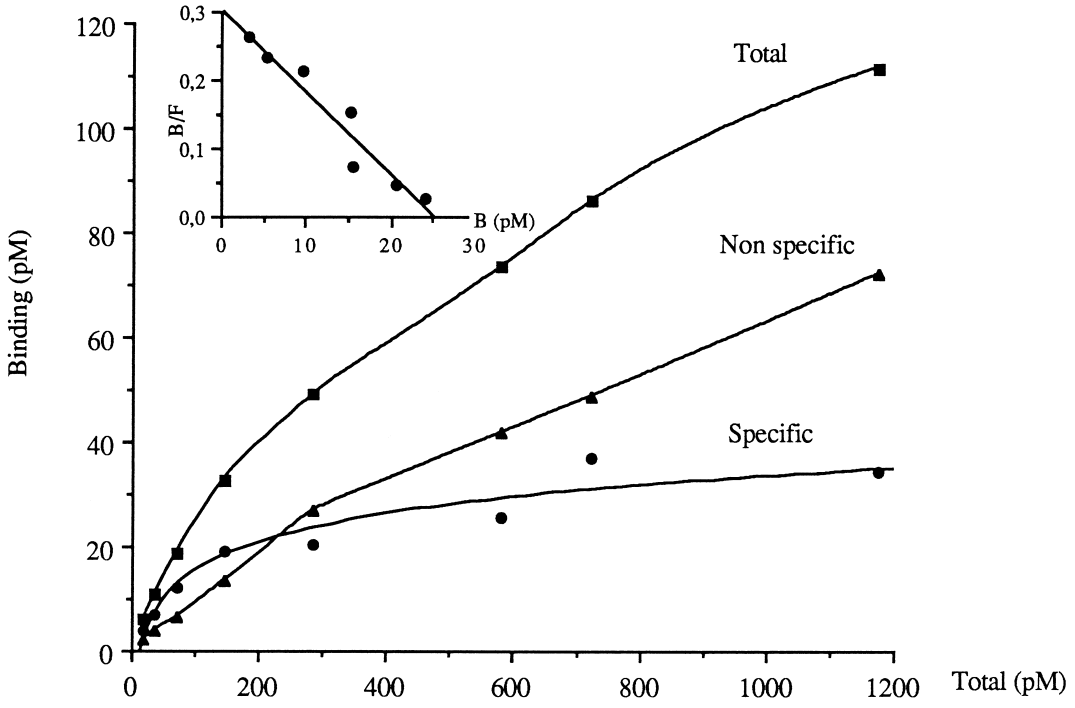

FIG. 3. Saturation curves and Scatchard analysis of [^{125}I]PE2I binding on rat striatal membranes. Striatal membranes were incubated with increasing concentrations of [^{125}I]PE2I (0.01–1.5 nM). Nonspecific binding was determined in the presence of 30 μM cocaine. Three independent experiments were performed, and this figure is one representative experiment.

and rapidly filtered through Whatman GF/B filters. The filters were washed twice with 3 mL ice-cold buffer, and the residual radioactivity was measured in a gamma scintillation counter (LKB 1282 Compugamma). The binding assays were run in duplicate. The specificity of PE2I has previously been studied by binding assays using tritiated ligands specific for each monoamine transporter (DA, 5HT and NE) (10).

For pharmacological characterization, [^{125}I]PE2I was incubated at a concentration of 0.08 nM in the same conditions as for saturation studies, with increasing concentrations of drugs known to bind to the DA, NE, and 5 HT transporters—respectively, GBR 12909, paroxetine, and nisoxetine. Total binding was determined in the absence of any inhibitor, and nonspecific binding was determined using 30 μM cocaine. Incubation was conducted and samples were treated as previously described.

Biodistribution Studies in Rats

For kinetic studies, rats received an IV injection of [^{125}I]PE2I (1 MBq in 250 μL of EtOH/saline 50/50) and were sacrificed 30 min, 1 h, 2 h, or 4 h postinjection (n = 5 to 6 rats per group). Samples of blood and cerebral regions (cerebellum, striatum, and frontal cortex) were removed and weighed, and their radioactivity was measured with a gamma scintillation counter (LKB 1282 Compugamma). The uptake was expressed as the percentage of injected dose per gram of tissue (%ID/g). To evaluate *in vivo* deiodination of the radiolabelled product, the entire thyroid gland of each animal was removed and its radioactivity measured.

Three groups of rats were used for blocking studies (n = 5 to 6 rats per group). Each group received an IV injection of saline, GBR 12909, at a dose of 5 mg/kg, or paroxetine at a dose of 5 mg/kg. All animals received 1 MBq of [^{125}I]PE2I at 30 min postinjection and were sacrificed 2 h later. The procedure described above was then repeated.

TABLE 1. Relative Potencies of Competitors for [^{125}I]PE2I Binding in the Rat Striatum

Competitor	Ki (nM)
GBR 12909	8 ± 2
Cocaine	500 ± 40
Paroxetine	>1000
Nisoxetine	>1000

Striatal membranes were incubated with [^{125}I]PE2I (0.08 nM) and with competitors at different concentrations. Results are expressed as mean inhibitory constants (K_i) ± SEM, and reflect three independent experiments.

Ex Vivo *Autoradiographic Studies*

Ex vivo autoradiographic studies were performed in male Wistar rats (200–250 g). Rats were injected with 0.3 mL 4 MBq of [^{125}I]PE2I IV. They were sacrificed 2 h postinjection by rapid decapitation. The brains were removed, rapidly frozen on dry ice and kept at −70°C until use. Sections 20-μm thick were cut at −18°C (Reichert-Jung, Cryocut 1800) and thaw-mounted on glass slides. Sections were placed in X-ray cassettes and were apposed to β_{max} hyperfilms (Amersham) for 4 weeks at room temperature. Autoradiograms were developed (Kodak LX24), fixed (Kodak AL4), and quantified using a computer imaging system (Biocom, France).

Biodistribution Studies in Monkeys

Experiments in monkeys were carried out in compliance with appropriate guidelines from the Ministère de l'Agriculture, France.

Biodistribution studies in monkeys were performed after IV injection of [^{123}I]PE2I, 75 MBq to 150 MBq in a 5-mL volume. Two

TABLE 2. Time Course of [^{125}I]-PE2I Concentration in Blood and Cerebral Areas After IV Injection in the Rat

	Concentration (% ID/g tissue)			
Tissue	**30 min**	**1 h**	**2 h**	**4 h**
Blood	0.207 ± 0.020	0.216 ± 0.019	0.191 ± 0.056	0.184 ± 0.015
Cerebellum	0.100 ± 0.015	0.055 ± 0.010	0.038 ± 0.010	0.021 ± 0.002
Striatum	1.061 ± 0.132	0.537 ± 0.102	0.272 ± 0.084	0.074 ± 0.03
Frontal cortex	0.124 ± 0.020	0.070 ± 0.016	0.039 ± 0.006	0.018 ± 0.004

Results are expressed as mean percentage of injected dose per gram of tissue ± SD; n = 5 to 6 animals per time point.

male cynomolgus monkeys weighing 4.5 kg were anesthetized with repeated IM injections of ketamine (Imalgene 500®, 5–10 mg/kg/h). The animals were placed in a supine position. The head was immobilized with adhesive tape and positioned at the center of the detection ring of a brain-dedicated camera, Ceraspect® (D.S.I. Waltham, MA). The spatial transaxial resolution was 8 mm at the center and 5 mm at the periphery. The scintigraphic recording started at the time of [^{123}I]PE2I injection and consisted of 5-min acquisitions over an 80-min period. Images were reconstructed using a back projection algorithm and a Butterworth filter (cut off: 0.95 cm, order: 10). The size of the reconstructed slice voxel was 1.67 mm × 1.67 mm × 1.67 mm. The three transaxial slices corresponding to the highest striatal uptake were added, yielding a final slice of 5-mm thickness. Regions of interest of right and left striata and the posterior cerebral cortex were drawn according to NMR imaging of the animal in the same position and with the same orientation of the head, using a Biospec® 1.5 tesla imager (Brücker). The NMR imager was connected to the Ceraspect® camera via an Ethernet® link. Striatal and extrastriatal regions of interest were drawn according to NMR images, and the counting rate in each region of interest was calculated at each acquisition time. Counting rate was expressed as total count in each region of interest and as the ratio between mean counting in the striatum and in the extrastriatal posterior cortex.

RESULTS

Radiolabelling

The stannyl precursor of PE2I obtained by a method previously described by us (9) provided radioiodinated PE2I ([^{125}I]PE2I or [^{123}I]PE2I) with a greater than 50% yield. After purification by HPLC, radioiodinated compounds were obtained with high radiochemical purity (>95%). As these compounds were obtained without carrier, they displayed a high specific activity of 75 and >185 TBq/mmol for [^{125}I]PE2I and [^{123}I]PE2I, respectively. Chemical identity was checked after labelling by co-injection on HPLC of PE2I in cold and radioactive forms (Fig. 2).

In Vitro *Binding Studies*

The affinity and density of specific [^{125}I]PE2I binding sites were determined using increasing concentrations of [^{125}I]PE2I (0.01 to 1.5 nM). Binding was saturable and had high affinity. Scatchard transformation of the data resulted in a linear relation, suggesting a one-site model (Hill coefficient = 1.01) with a Kd of 0.09 ± 0.01 nM (mean ± SE) and a B_{max} of 280 ± 44 fmol/mg protein (Fig. 3).

The specific binding of [^{125}I]PE2I (0.09 nM) was studied in rat striatal membranes in competition with other ligands of DA, 5HT, and NE uptake sites (Table 1). In rat striatal membranes, inhibitors of DA uptake such as GBR 12909 and cocaine are competitors for [^{125}I]PE2I binding, with K_i values of 8 ± 2 nM and 500 ± 40 nM, respectively. Paroxetine, a specific 5HT uptake inhibitor, did not inhibit binding of [^{125}I]PE2I in rat striatal membranes (K_i >1000 nM), and similarly nisoxetine, a selective norepinephrine uptake inhibitor with high affinity for this site, did not inhibit binding at all (K_i >1000 nM).

Biodistribution Studies in Rats

Thyroid radioactivity was expressed as the percentage of injected dose per whole gland (%ID), as the net weight of this gland is difficult to measure with precision. The radioactivity for [^{125}I]PE2I showed a gradual increase from 0.27 ± 0.11% ID at 30 min to 2.29 ± 0.54 at 4 h postinjection. The time course of the concentration of [^{125}I]PE2I in the blood and in different regions of the brain is shown in Table 2. Whole-blood radioactivity exhibited stable values, about 0.2%, 30 min to 4 h postinjection. The highest accumulation of [^{125}I]PE2I was observed in the striatum compared to the frontal cortex and the cerebellum. Radioactivity in the striatum was 1.06 ± 0.13% ID/g tissue at 30 min and was decreased by 49%, 74%, and 93% at 1 h, 2 h, and 4 h, respectively. In contrast, the frontal cortex and cerebellum displayed rapid washout, providing a very weak degree of radioactivity from 1 h postinjection.

As shown in Figure 4, a preinjection of GBR 12909 (5 mg/kg) induced a strong blocking effect (−74%) in the striatum, whereas this preinjection had no effect on tracer binding in the cerebellum and frontal cortex. Moreover, preinjection of paroxetine (5 mg/kg) had no effect on uptake of [^{125}I]PE2I, whatever the tissue.

Ex Vivo *Autoradiographic Studies*

Autoradiographic studies in normal rats showed a high accumulation of [^{125}I]PE2I in cerebral areas rich in dopamine transporters, in particular the striatum, accumbens nucleus, and olfactory tubercles (Fig. 5a). We also observed (Fig. 5b) binding of PE2I in the substantia nigra on another slice. By contrast, brain regions rich in 5HT transporters (thalamus, cerebral cortex) were not visualized.

Biodistribution Studies in Monkeys

Figure 6 shows a representative transaxial image acquired at 1 h postinjection. There was a high and symmetrical accumulation of radioactivity in the striatum; by contrast, a very low uptake was observed in the cortex. The quantitative relationship between uptake and time (Fig. 7) shows that an equilibrium was obtained about 1 h postinjection, with a ratio between striatum and posterior cerebral cortex of 3.5 to 4.5.

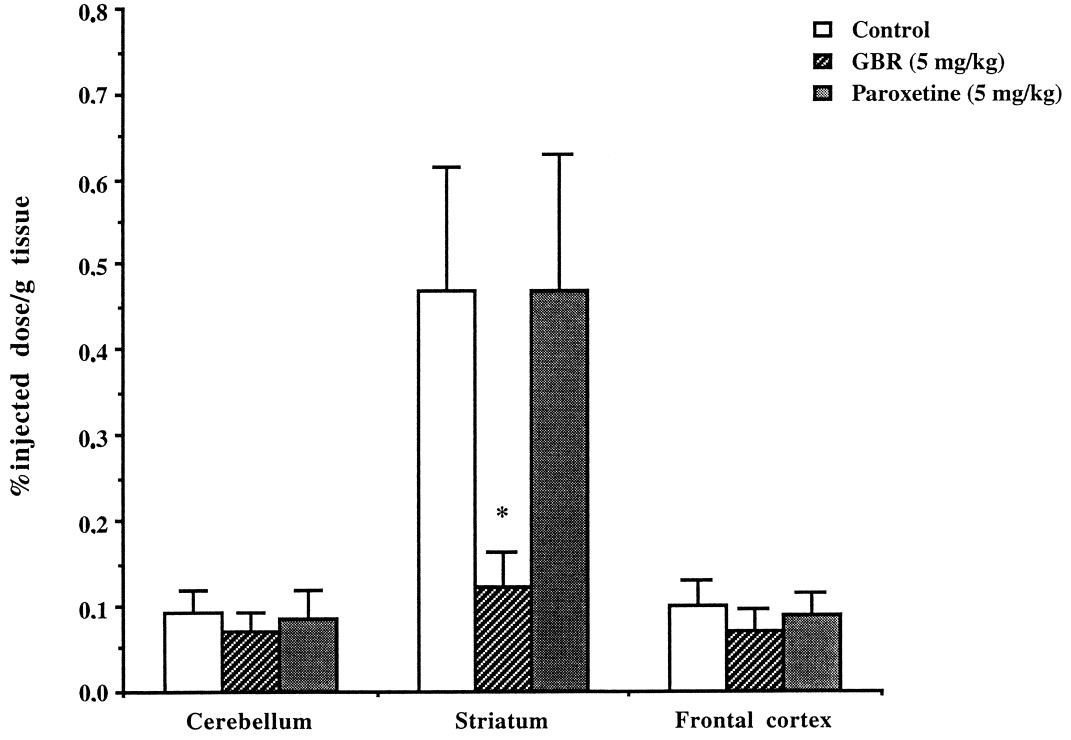

FIG. 4. Cerebral biodistribution of [^{125}I]PE2I in rats: saturation studies. Animals received an IV injection of saline, GBR12909, or paroxetine 30 min before [^{125}I]PE2I injection and were sacrificed 2 h later. n = 5–6 rats per group. *$p < 0.05$, t-test for unpaired values.

DISCUSSION

The loss of neurons accompanying some neurodegenerative diseases such as Parkinson's can be explored by dopamine transporter imaging. For this, Boja *et al.* proposed a cocaine congener, RTI-55 (3). This promising ligand, also named βCIT, has been used in humans, and several teams employing it have observed a reduction in the uptake of this ligand in the caudate putamen of sufferers of Parkinson's disease (1, 15). However, βCIT does not fulfil all the criteria of specificity and kinetics for wide use in nuclear medicine. To improve βCIT characteristics for *in vivo* human use, several groups have proposed new cocaine congeners such as [^{123}I]IPT (14, 19, 22) and [^{123}I]FP-CIT (16, 18, 23), but none of these have optimum specific and kinetic properties. Thus, we have synthetized a series of cocaine derivatives and selected a promising structure, (*E*)-*N*-(3-iodoprop-2-enyl)-2β-carbomethoxy-3β-(4′-methylphenyl)nortropane, with a combination of aromatic and nitrogen substitution (10).

Radiolabelled [^{125}I]PE2I and [^{123}I]PE2I were obtained with high yields and high radiochemical purity from the stannyl precursor. The good results obtained with this radiolabelling method are in accordance with results previously reported by Goodman *et al.* (14). This labelling method is therefore suitable in the preparation of radiotracers for human use. *In vitro* [^{125}I]PE2I binding to the dopamine transporter was evaluated on rat striatal membranes. These experiments demonstrated that [^{125}I]PE2I had a high affinity for the dopamine transporter, with a Kd of 0.09 ± 0.01 nM and a

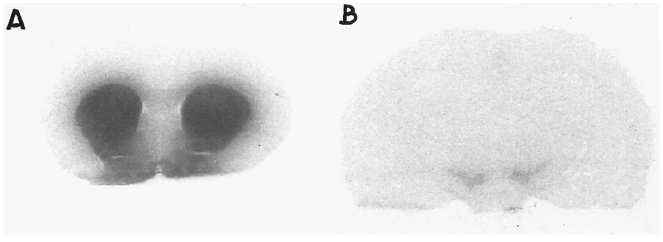

FIG. 5. Cerebral biodistribution of [^{125}I]PE2I in rats: autoradiographic studies. Animals received an IV injection of [^{125}I]PE2I and were sacrificed 2 h later. High accumulation was observed in the striatum, accumbens nuclei, and olfactory tubercles (A), and also in the substantia nigra (B).

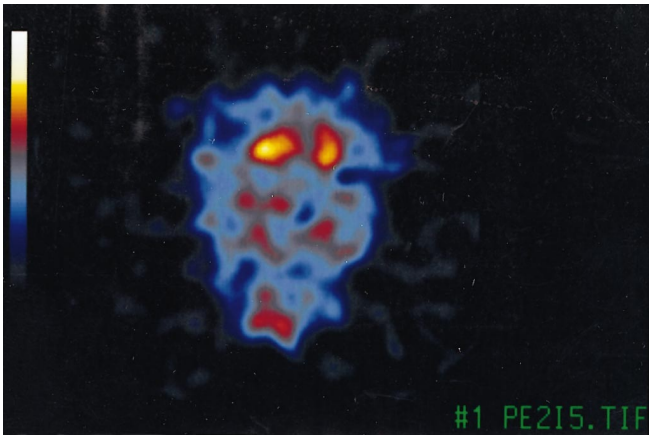

FIG. 6. SPECT Imaging in a cynomolgus monkey 1 h postinjection of [^{123}I]PE2I. High accumulation was observed in the striatum.

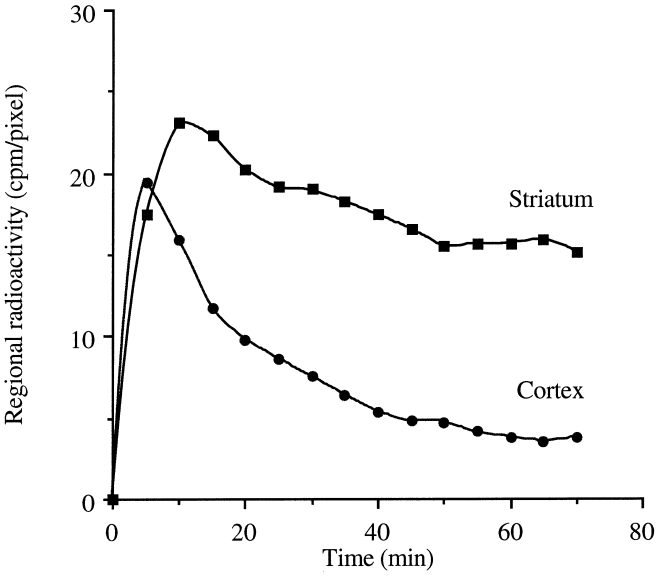

FIG. 7. Striatal and cortical uptake of [^{123}I]PE2I in a cynomolgus monkey: kinetics studies. Two experiments were performed, and this figure is one representative experiment.

B_{max} of 280 ± 44 fmol/mg protein. The affinity is of the same order as the affinity of other cocaine derivatives such as βCIT (Kd = 0.12 nM) (3) and IPT (Kd = 0.25 nM) (19).

The specificity of PE2I has been evaluated in a previous work (10) by *in vitro* competition between PE2I and specific tritiated ligands of monoamine transporters on rat striatal and cortex membranes. We observed dopamine-to-serotonin selectivity ratio of 29 for PE2I, whereas ratios of 0.1, 1.7, and 7 have been obtained for βCIT (9), βCDIT (13), and IPT (19), respectively. This is in agreement with results reported here, with [^{125}I]PE2I showing considerable competition between PE2I and GBR 12909, whereas paroxetine and nisoxetine did not compete with PE2I. This demonstrates high specific binding of PE2I to the dopamine transporter. It seems, therefore, that the combination of nitrogen and phenyl substitution gives PE2I high *in vitro* affinity and specificity for the dopamine transporter compared to other tropane derivatives.

In vivo experiments in rats showed a very high and rapid accumulation of [^{125}I]PE2I in the striatum and substantia nigra, both rich in dopamine transporters, whereas very low fixation was observed in areas rich in serotonin transporters such as the frontal cortex. In addition, striatal accumulation was dramatically decreased with a preinjection of GBR 12909, whereas no modification was observed with a preinjection of paroxetine, demonstrating a high *in vivo* specificity to the dopamine transporter.

Kinetic studies in the rat brain showed that a maximal striatum/cerebellum ratio of 10 was obtained during the first 30 min postinjection, which then plateaued for more than 1 h. These findings led us to study *in vivo* cerebral distribution of [^{123}I]PE2I in the nonhuman primate. A rapid uptake in the striatum, with a high contrast compared to cortical areas, was observed in cynomolgus monkeys. Moreover, the striatal accumulation seemed to reach a plateau between 30 and 80 min postinjection. This *in vivo* brain biodistribution has recently been confirmed using PET with [^{11}C]-labelled PE2I (C. Halldin, personal communication).

Very recently, Elmaleh *et al.* (8) described another cocaine congener, [^{125}I]IACFT, a closed structural analogue of PE2I. This compound seemed to display valuable properties of kinetics and specificity (11), but to date it is difficult to compare the respective properties of IACFT and PE2I owing to the difference in protocols. Indeed, IACFT has been evaluated *in vitro* in monkey striatal membranes, whereas we performed the evaluation on rat membranes.

An interesting study by Ishikawa *et al.* (16) demonstrated that FP-βCIT in Parkinson patients gave the same results as the reference PET method using [^{18}F]DOPA. Indeed, both methods using a striatum-to-occipital cortex ratio were able to distinguish between normal volunteers and Parkinson patients. We therefore propose that SPECT imaging, using a tracer such as PE2I with similar kinetics to FP-βCIT but with higher specificity for the dopamine transporter, will provide very valuable information. Moreover, as PE2I can be labelled with C-11, we have a versatile ligand for PET and SPECT, with exactly the same structure and similar kinetics and specificity. It can therefore be assumed that PET would be very useful to develop and check SPECT quantification.

In conclusion, we have developed PE2I, a new tracer for dopamine transporter exploration, which can be easily obtained in nuclear medicine centers. PE2I appears to date to be the most specific iodinated ligand for the dopamine transporter, with good kinetics for *in vivo* human exploration.

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