Endogenous Dopamine (DA) Competes With the Binding of a Radiolabeled D_3 Receptor Partial Agonist In Vivo: A Positron Emission Tomography Study

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ABSTRACT A series of microPET imaging studies were conducted in anesthetized rhesus monkeys using the dopamine D_3-selective partial agonist, [^{18}F]**5**. There was variable uptake in regions of brain known to express a high density of D_3 receptors under baseline conditions. Pretreatment with lorazepam (1 mg/kg, i.v. 30 min) to reduce endogenous dopamine activity before tracer injection resulted in a dramatic increase in uptake in the caudate, putamen, and thalamus, and an increase in the binding potential (BP) values, a measure of D_3 receptor binding in vivo. These data indicate that there is a high level of competition between [^{18}F]**5** and endogenous dopamine for D_3 receptors in vivo. **Synapse 65:724–732, 2011.** © 2010 Wiley-Liss, Inc.

INTRODUCTION

Alterations in the dopaminergic pathways are thought to be involved in the etiology of a variety of neurological and neuropsychiatric disorders, including Parkinson's Disease, dystonia, and schizophrenia (Jardemark et al., 2002; Kapur and Mamo, 2003; Karimi et al., in press; Korczyn, 2003; Lee et al., 1978; Luedtke and Mach, 2003; Missale et al., 1998; Nieoullon, 2002; Perlmutter et al., 1997). In addition, activation of the dopaminergic pathways may mediate the reinforcing effects of pyschostimulants, including cocaine and amphetamines (Nader et al., 1999; Uhl et al., 1998; Volkow et al., 2002).

Molecular genetic studies have defined two types of dopamine receptors, the D_1-like (D_1 and D_5 receptor subtypes) and the D_2-like (D_2, D_3, and D_4 receptor subtypes), based upon structural and pharmacological similarities. D_1-like receptors are structurally similar and positively linked to the activation of adenylyl cyclase via coupling to the $G_{alpha(S)}/G_{alpha(olf)}$ class of G proteins (Herve et al., 2001). Stimulation of the D_2-like receptors results in the coupling with the G_i/G_o class of G proteins, leading to the inhibition of adenylyl cyclase activity (Sealfon and Olanow, 2000; Sibley and Monsma, 1992; Vallone et al., 2000).

There is approximately 78% amino acid homology in the transmembrane spanning (TMS) regions of the D_2 and D_3 receptors that comprise the ligand binding site of these receptors (Sokoloff et al., 1990). Despite the similarities in the structure of the D_2 and D_3 receptors, the D_2 and D_3 receptors differ in their (a) neuroanatomical localization, (b) levels of receptor expression, (c) efficacy in response to agonist stimulation, and (d) regulation and desensitization (Joyce, 2001; Luedtke and Mach, 2003; Mach et al., 2004). Because of the high localization of D_3 receptors in limbic structures of the brain and the possibility that abnormalities in striatal D_3 receptors contribute to the pathophysiology of dystonia, there has been a concerted effort to develop D_3-selective radiotracers for imaging the expression of this receptor in a variety of CNS disorders.

Previous efforts to image the D_3 receptor have focused on the preparation of radiolabeled analogs of the conformationally-flexible benzamide analogs including, RGH-1756 (Langer et al., 2000; Sovago

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et al., 2005), the benzothiophene-2-carboxamide analog **1** (Kuhnast et al., 2006), the benzofuran-2-carboxamide analog **2** (Turolla et al., 2005), and [^{18}F]**3**, which is a 2-fluoroethoxy analog of FAUC 356 (Hocke et al., 2008). However, the low uptake of these compounds in regions of the brain known to express D_3 receptors was attributed to the high lipophilicity of these analogs, which could cause low uptake in the CNS and high level of nonspecific binding for the amount of the radiotracer that does cross the blood–brain barrier. Recently, a ^{11}C-labeled imidazolidinone analog, [^{11}C]**4**, was prepared and evaluated in vivo. This compound has a subnanomolar affinity for D_3 receptors and a 100-fold selectivity for D_3 versus D_2 receptors. The log *P* value of this compound is 2.9, which means that it should readily cross the blood-brain barrier (Waterhouse, 2003). Unfortunately, in vivo imaging in rhesus monkeys revealed no specific binding to D_3 receptors (Bennacef et al., 2009).

In this study, we report the synthesis of [^{18}F]*N*-[4-[4-(2-(2-fluoroethoxy)phenyl)piperazine-1-yl]butyl]-4-(3-thienyl)benzamide, [^{18}F]**5**, a radiolabeled D_3 partial agonist that is capable of imaging the dopamine D_3 receptor in vivo with positron emission tomography (PET). We also report that there is a high level of competition between [^{18}F]**5** and endogenous dopamine for D_3 receptors. However, D_3 dopamine receptors can be efficiently and reproducibly imaged following the administration of the benzodiazepine agonist, lorazepam, which transiently reduces synaptic dopamine levels (Dewey et al., 1992; Nader et al., 2006).

MATERIALS AND METHODS

Radiosynthesis

The synthesis of the mesylate precursor, **7**, and unlabeled **5** (high-performance liquid chromatography (HPLC) standard) will be published separately. [^{18}F]**5** was prepared via displacement of the corresponding mesylate precursor with [^{18}F]potassium fluoride/Kryptofix 2.2.2 in DMSO at 80°C for 10 min. The final product was purified by reversed-phase HPLC (C-18 column; 52% methanol: 48% ammonium formate buffer) in 50% yield and a specific activity of ~1500 Ci/mmol.

Receptor binding assays

In vitro binding assays were conducted using the assay conditions described by Chu et al. (2005). The radioligand used in the binding assay was [^{125}I]IABN, which has a high affinity for dopamine D_2, D_3, and D_4 receptors (Luedtke et al., 2000).

Intrinsic activity assay

The intrinsic activity at dopamine D_3 receptors was determined using the cAMP assay conditions as described by Chu et al. (2005). In this assay, quinpirole was used as a reference full agonist at both D_2 and D_3 dopamine receptors.

PET data acquisition

MicroPET imaging studies were conducted on a Focus 220 microPET scanner (Siemens Medical Systems, Knoxville, TN). Male rhesus monkeys (8–12 kg) were initially anesthetized with ketamine (10–15 mg/kg) and injected with glycopyrrolate (0.013–0.017 mg/kg) to reduce saliva secretions. PET tracers were administered ~90 min after ketamine injection. Subjects were intubated and placed on the scanner bed with a circulating warm water blanket and blankets. A water-soluble ophthalmic ointment was placed in the eyes, and the head was positioned in the center of the field using gauze rolls taped in place. Anesthesia was maintained with isoflurane (1.0–1.75% in 1.5 L/min oxygen flow). Respiration rate, pulse, oxygen saturation, body temperature, and inspired/exhaled gasses were monitored throughout the study. Radiotracers and fluids were administered using a catheter placed percutaneously in a peripheral vein. For the metabolism studies, a catheter was placed percutaneously in the contralateral femoral artery to permit the collection of arterial blood samples and for the determination of the blood time-activity-curve using a continuous flow detection system (Hutchins et al., 1986). In each microPET scanning session, the head was positioned supine with the brain in the center of the field of view. A 10-min transmission scan was performed to check positioning; once confirmed, a 45-min transmission scan was obtained for attenuation correction. Subsequently, a 120-min dynamic emission scan was acquired after administration of ~5 mCi of [^{18}F]**5** via the venous catheter. For the lorazepam studies, animals were given an intravenous dose of the drug (1 mg/kg in saline) approximately 30 min before injection of the radiotracer. Blocking studies were also conducted in lorazepam-treated animals by administering compound **6** (1 mg/kg, i.v.), a structurally-related benzamide analog (Fig 1), 5 min before tracer injection.

Time activity curves and metabolite analysis

Time activity curves for the initial 5 min postinjection were determined using the continuous flow detection system attached to a percutaneous arterial catheter. Arterial blood samples for metabolite analysis were taken in a heparinized syringe from the same catheter at 5, 15, 30, and 60 min post injection. The 5-min sample was taken immediately after the pump for the detector was turned off; the arterial line was flushed before collection of subsequent samples. Additional blood samples were taken at 45, 90, and 120 min for the TAC (Fig. 4C). Metabolite analysis was

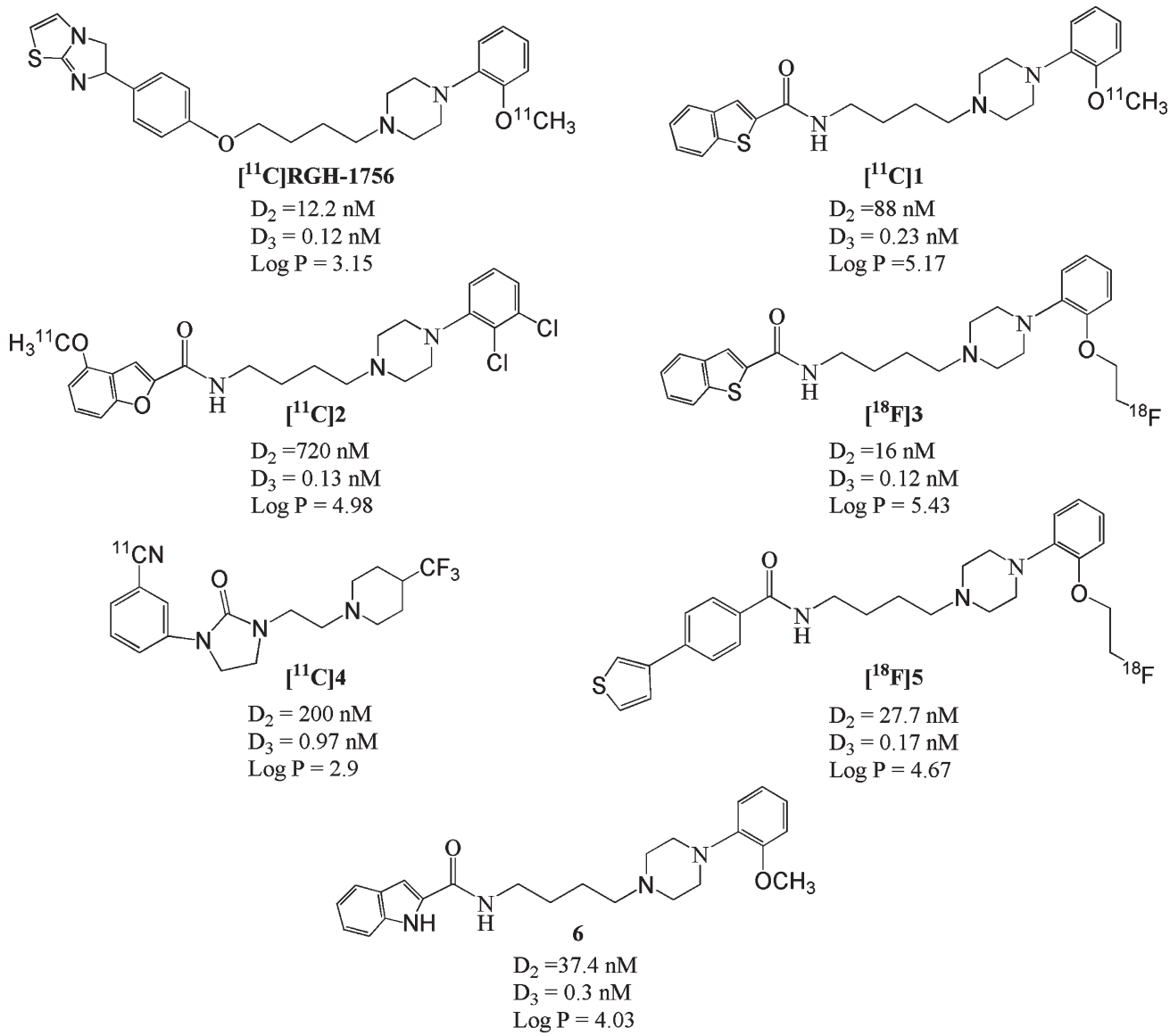

Fig. 1. Structures of D_3-selective ligands reported in the literature.

performed using a solid-phase extraction technique previously used for similar studies (Mach et al., 1997). A 1-mL aliquot of whole blood was centrifuged to separate plasma from packed red cells. Each fraction was counted, a 400-μL aliquot of plasma was removed, counted and deproteinated with 6 mL of 2: 1 methanol: 0.4 M perchloric acid mixture. The supernatant was diluted in 4 mL water and applied to an activated C-18 light Sep-Pak. The cartridge was neutralized with 2 mL 1N NaOH, then rinsed twice with water, and extracted with two portions of methanol (2 mL, 1 mL). All samples were counted in a Ludlum well counter. The methanol extracts were combined, concentrated in vacuo and rediluted to 150 μL of methanol for injection onto the HPLC. HPLC analysis was performed using a reversed-phase Phenomenex analytical column (Prodigy 250 × 4.6) with a mobile phase of methanol: 0.1 M ammonium formate buffer, pH 4.5 60:40. The flow rate was 0.8 mL/min, 0.5 min/fraction and 36 fractions were collected and counted or each sample. The location of the parent UV peak was determined by injection of cold standard. The purity and stability of the injectate were confirmed by analysis of an in vitro control (reserved injectate added to a prescan blood sample and processed as described above after all the ex vivo samples were completed). This control also confirmed the stability of the radiotracer under the conditions used to process the blood for metabolite analysis. The percentages of unchanged parent compound and its metabolites were determined by decay correcting the counts and dividing the amount of recovered activity in all

TABLE I. In vitro binding data and intrinsic activity (IA) for compound 5[a,b]

D_{2long}	D_3	D_4	D_2:D_3 ratio	%IA D_2	%IA D_3
27.7 ± 5.4 nM	0.17 ± 0.01 nM	246 ± 13.3 nM	163	29.3 ± 7.3	34.5 ± 1.7

[a]K_i values (nM) were determined using human receptors expressed in HEK cells with ^{125}I-IABN. The K_i values represent the mean values for $n > 3$ determinations.
[b]Percent intrinsic activity (%IA) at human D_2 or D_3 receptors was determined using a forskolin-dependent adenylyl cyclase whole cell assay at a concentration of test compound $< 10 \times K_i$ value. The mean values ($n > 3$) were normalized to values obtained using the full agonist quinpirole.

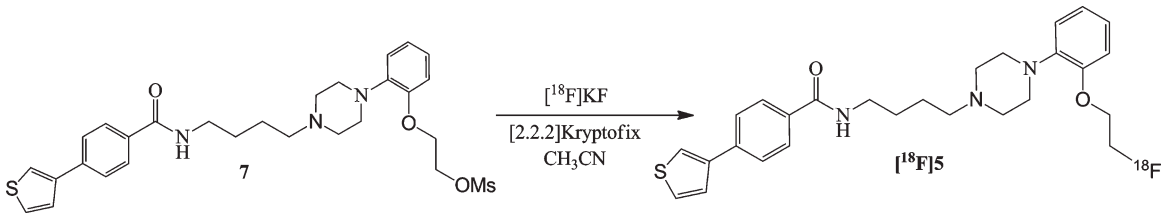

Fig. 2. Radiosynthesis of [^{18}F]**5**.

samples and multiplying by 100. (Table II) Only a single peak for the parent compound was observed in the in vitro control and >95% of the activity was recovered.

Image processing and analysis

Acquired list mode data were histogrammed into a three-dimensional set of sinograms and binned to the following time frames: 3 × 1 min, 4 × 2 min, 3 × 3 min and 20 × 5 min. Sinogram data were corrected for attenuation and scatter. Maximum a posteriori (MAP) reconstructions were done with 18 iterations $\beta = 0$. A 1.5 mm Gaussian filter was applied to smooth each MAP reconstructed image. These images were then coregistered with MRI images to identify the regions of interest with AnalyzeDirect software (AnalyzeDirect, Inc., Overland Park, KS). Three-dimensional regions of interest were manually drawn through all planes of coregistered MRI images for the caudate, putamen, and cerebellum. The regions of interest were then overlaid on all reconstructed PET images to obtain time-activity curves. Activity measures are expressed as %ID/c.c. (Figs. 4A and 4B).

RESULTS

In vitro binding studies indicate that **5** has a high affinity for D_3 receptors (0.17 nM) and ~160-fold selectivity for D_3 versus D_2 receptors (Table I). Functional assays demonstrated that **5** partially inhibited forskolin-dependent adenylyl cyclase activity relative to quinpirole (~35% maximal response) in CHO cells transfected with hD_3 receptor (Table I), indicating that it is a partial agonist at D_3 receptors. These data are similar to that previously reported by our group for a diverse panel of *N*-phenyl piperazine analogs structurally similar to **5** (Chu et al., 2005).

The synthesis of [^{18}F]**5** was achieved in approximately 60 min in an overall radiochemical yield of 50% from starting [^{18}F]fluoride. The specific activity of the final compound was >1500 mCi/μmol (end of synthesis), which is suitable for microPET imaging studies (Fig. 2).

MicroPET studies were conducted in rhesus monkeys under 1% isoflurane anesthesia ($n = 4$). These initial microPET imaging studies yielded variable results (Fig. 3), with one study demonstrating a high uptake in the caudate and putamen (monkey 4), but the other studies (monkeys 1–3) indicating no specific uptake of the radiotracer in regions of brain known to express D_3 receptors (Xu et al., 2009, 2010). To determine if endogenous dopamine may be responsible for the variability of the PET results, a second series of studies were conducted in which the animal received an i.v. dose of lorazepam (1.0 mg/kg) 30 min before the injection of the radiotracer. Lorazepam was previously shown to increase striatal [^{11}C]raclopride binding (Dewey et al., 1992), and we previously used this paradigm in our evaluation of the effect of endogenous dopamine to interfere with the binding of the $D_{2/3}$ radiotracer, [^{18}F]FCP, in PET studies in rhesus monkeys (Nader et al., 2006). The results of this study indicate that pretreatment with lorazepam eliminates the between-subject variability of the uptake of [^{18}F]**5** in regions of the brain known to express D_3 receptors (Fig. 3). There was also a notable increase in [^{18}F]**5** in the thalamus, a region of brain known to express D_3 versus D_2 receptors (Rabiner et al., 2009). The tissue-time activity curves in the lorazepam-treated animals relative to the baseline study, demonstrated a dramatic increase in D_3 receptor availability as measured by the Logan DVR-1 analyses (Fig. 4) (Logan, 2000). Lorazepam had no effect on the blood curve (Fig. 4) or metabolism (Table II) of [^{18}F]**5**. This indicates that the increases seen in

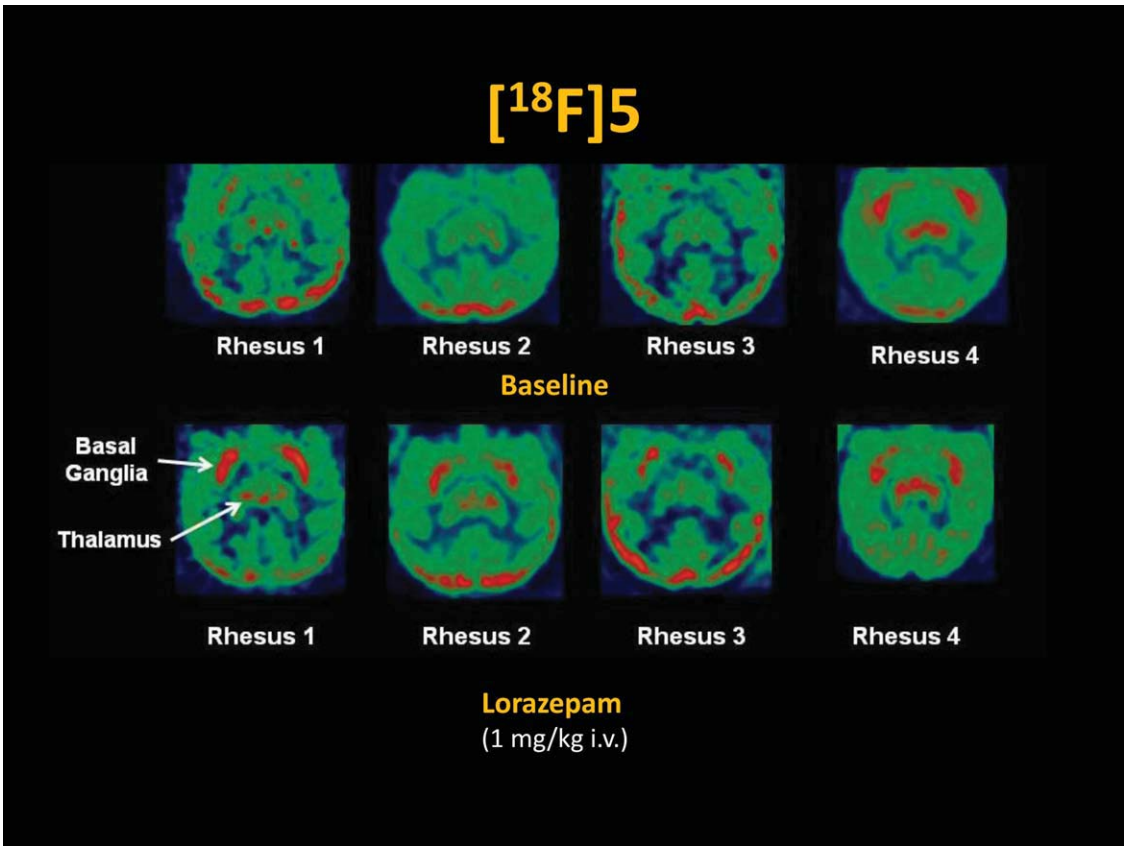

Fig. 3. MicroPET imaging studies of [^{18}F]**5** in rhesus monkeys.

the brain were not the result of a change in the input function of the parent compound. Blocking studies with compound **6**, a ligand having a high affinity and selectivity for D_3 versus D_2 receptors (Fig. 1), are consistent with [^{18}F]**5** labeling D_3 versus D_2 receptors in vivo (Fig. 5).

DISCUSSION

The development of PET radiotracers having a high selectivity for dopamine D_3 versus D_2 receptors has been an active area of research in recent years. Although a number of D_3 selective radiotracers, labeled with either carbon-11 (t = 20.4 min) or fluorine-18 (t = 110 min), have been reported over the past decade, none have proven to be useful for imaging this receptor in vivo. Consequently, studies aimed at imaging the D_3 receptor must resort to using nonselective, high D_2/D_3 affinity radiotracers such as the radiolabeled antagonists [^{11}C]raclopride (Volkow et al., 2008; Yokoi et al., 2002), [^{11}C]fallypride or [^{18}F]fallypride (Buchsbaum et al., 2006; Narendran et al., 2004, 2009) and [^{11}C]FLB457 (Cselenyi et al., 2006; Vandehey et al., 2010), and full agonists at D_2 and D_3 receptors, [^{11}C](+)-PHNO (Ginovart et al., 2006; Narendran et al., 2006), and [^{11}C]NPA (Hwang et al., 2000; Narendran et al., 2004). Although methods have been developed to separate the "D_3 signal" from the "D_2 signal" (Rabiner et al., 2009), radiotracers capable of imaging the D_3 receptor without interference from the labeling of D_2 receptors are clearly needed for in vivo studies with PET.

In the current study, we present evidence demonstrating that [^{18}F]**5** is a potential radiotracer for imaging D_3 receptors in vivo with PET. [^{18}F]**5** has a subnanomolar affinity for D_3 receptors and a 160-fold higher affinity for D_3 compared with D_2 dopamine receptors. An important observation in the current study is the ability of endogenous dopamine to compete with D_3 receptors in vivo to the extent that it is necessary to decrease synaptic levels of dopamine by pretreatment with lorazepam to image D_3 receptors in the anesthetized rhesus monkey. This observation is consistent with previous reports regarding the high in vivo occupancy of D_3 receptors by endogenous dopamine, including (1) the binding of [^{125}I]iodosuplride to D_3 receptors in autoradiography studies requires either extensive washing of tissue slices to remove endogenous dopamine, or the in vivo depletion of monoamines with tetrabenazine before euthanasia and tissue preparation (Schotte et al., 1992, 1996) and (2) endogenous dopamine protects the D_3 receptor from

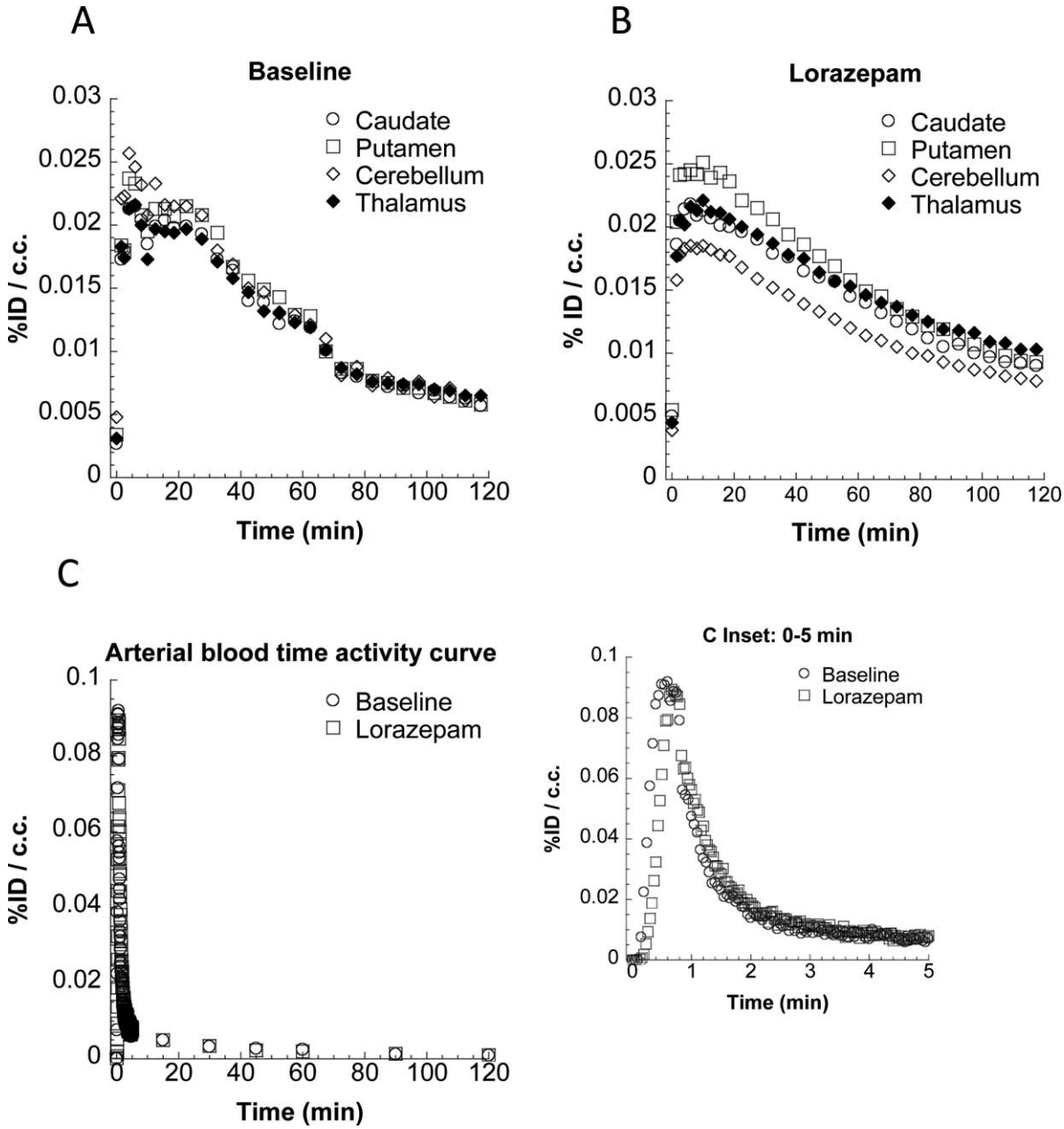

Fig. 4. Blood and Tissue time-activity curves (TACs) of [^{18}F]**5** in from microPET imaging studies. **A** and **B**: Regional brain TACs from baseline and lorazepam studies. The uptake of [^{18}F]**5** in the representative monkey brain regions (caudate, putamen, cerebellum, and thalamus) reached peak accumulation in the caudate and putamen 5 min post i.v. injection. Lorazepam (1 mg/kg/i.v.) treatment increased [^{18}F]**5** uptake in the caudate, putamen, and thalamus. **C**: Lorazepam treatment did not change the arterial blood TAC compared with baseline data. The inset graph shows that there was also no change during the initial 5 min.

TABLE II. Percent parent compound in arterial blood samples

	% Parent compound	
Time (min)	Baseline	Lorazepam[a]
5	27.9 ± 4.5	33.6 ± 3.3
15	15.8 ± 1.7	16.7 ± 4.6
30	12.7 ± 4.7	11.4 ± 1.4
60	7.9 ± 3.3	4.2 ± 2.8

[a]30 min pretreatment, 1 mg/kg/i.v.

alkylation by 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) and the spiperone analog, *N*-(*p*-isothiocyanatophenethyl)spiperone (NIPS), in vivo whereas no such protection was observed at D_2 receptors (Levant, 1995; Zhang et al., 1999). Finally, several in vitro binding studies have revealed that dopamine has a higher affinity for cloned D_3 versus D_2 receptors (reviewed in Levant, 1997), and supports a

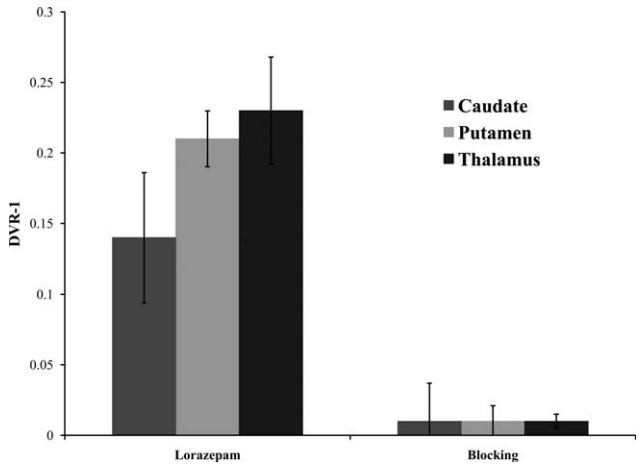

Fig. 5. BP analysis in caudate, putamen, and thalamus of microPET scans from baseline lorazepam treatment and blocking studies (under lorazepam). Blocking with Compound **6** (1 mg/kg/i.v.) significantly decreased [^{18}F]**5** BP in striatal regions: caudate ($P < 0.05$), putamen ($P < 0.001$) and extra-striatal regions: thalamus ($P < 0.005$).

greater occupancy of D_3 receptors by endogenous dopamine in vivo.

An unresolved question from the current study relates to the ability to image D_3 receptors in vivo without the need to deplete the dopaminergic synapse of endogenous neurotransmitter. It must be stressed that the current study was conducted under conditions of isoflurane anesthesia. Previous studies by Votaw et al. have shown that isoflurane increases the trafficking of the dopamine transporter (DAT) from the plasma membrane to the cell interior (Votaw et al., 2003, 2004), which may contribute to the increase in extracellular dopamine concentrations measured in microdialysis studies (Adachi et al., 2005; Tsukada et al., 1999). Potentiation of GABAergic neurotransmission by pretreatment with lorazepam decreases activity of dopaminergic neurons (Dewey et al., 1992). Consequently, lorazepam should minimize the effect of isoflurane-induced DAT internalization and prevent increased synaptic dopamine levels, thereby leading to an increase in the availability of D_3 receptors for labeling with [^{18}F]**5**. It is unlikely that all D_3 receptors in the CNS are occupied by endogenous DA. If this were the case, it would impair imaging D_3 receptors using the nonselective D_2/D_3 radiotracers such as [^{18}F]fallypride and [^{11}C](+)-PHNO. It is important to note that both [^{18}F]fallypride (a D_2/D_3 antagonist) and [^{11}C](+)-PHNO (a D_2/D_3 full agonist) are capable of imaging extrastriatal D_3 receptors in isoflurane-anesthetized nonhuman primate brain (Christian et al., 2000; Rabiner et al., 2009). In fact, one study reported that isoflurane anesthesia increased the binding of [^{11}C](+)-PHNO to striatal D_2/D_3 receptors in Sprague-Dawley rats relative to awake animals (McCormick et al., 2006). Further studies are clearly needed, in either awake rhesus monkeys or in translational imaging studies in human subjects, to determine the ability of [^{18}F]**5** to image D_3 receptors under conditions where physiological levels synaptic dopamine are present.

In summary, the results of the current study indicate that it is possible to image dopamine D_3 receptors in vivo with PET with [^{18}F]**5** even though this has not been demonstrated with structurally-related radiotracers. It should be noted that all of the radiotracers shown in Figure 1 have a similar high (i.e., subnanomolar) affinity for D_3 receptors and high selectivity for D_3 versus D_2 receptors (>100-fold). The key step taken in the current study was the use of lorazepam to reduce synaptic dopamine levels under conditions of isoflurane anesthesia, which should minimize competition between endogenous dopamine and [^{18}F]**5** for D_3 receptors and increase D_3 receptor availability. Additional studies are ongoing to determine if this effect is observed with other, high affinity D_3-selective radiotracers developed by our group.

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