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Synthesis and pre-clinical evaluation of a potential radiotracer for PET imaging of the dopamine D₃ receptor

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There is considerable interest in using positron emission tomography (PET) imaging to understand the function of dopamine D₃ receptors. Due to high sequence homology with D₂ receptors, development of D₃-selective PET radiotracers has been challenging. In an effort to overcome this issue, we report the radiosynthesis of a new selective D₃ ligand with carbon-11 (¹¹C]**1**), and its initial preclinical evaluation as a potential PET radiotracer for *in vivo* imaging of D₃ receptors. [¹¹C]**1** was prepared *via* [¹¹C]CO₂ fixation in 0.1% non-corrected radiochemical yield, good radiochemical purity (>95%) and high specific activity (>2000 Ci mmol⁻¹). [¹¹C]**1** exhibited specific binding to D₃ receptors using *ex vivo* autoradiography experiments with rat brain, but only 14-fold selectivity over D₂ receptors which is lower than the 1400-fold value reported previously for cell studies. Rodent PET imaging revealed reasonable uptake of the radiotracer in areas of the brain known to be rich in D₃ receptors.

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Introduction

Dopaminergic neurotransmission is mediated by activation of both pre- and post-synaptic dopamine receptors. There are five dopamine receptors which are G protein-coupled receptors (GPCRs) that are typically grouped into two subtypes: D₁-like and D₂-like receptors. The D₁-like family consists of D₁ and D₅ receptors, while the D₂-like family contains the D₂, D₃ and D₄ receptors. Through its action on these receptors, dopamine regulates CNS functions in a neuromodulatory fashion, and is involved in everything from motor control to hormone release, pain processing, and reward mechanisms.¹

The dopamine D₃ receptor was cloned and characterized by Sokoloff and coworkers in 1990.^{2,3} They described a distinct distribution in limbic areas of the brain, such as the nucleus accumbens and olfactory tubercle, and in further work have identified the D₃ receptor as an important target for clinical agents used to treat many dopaminergic disorders.⁴ In light of these findings, D₃ receptors are a large focus of current drug development efforts around dopamine-related disorders. For example, aripiprazole is an FDA-approved partial agonist for dopamine D₃ receptors that is used in the treatment of schizophrenia and manic depression.⁵

Despite these important findings, there still remains an incomplete understanding of the dopamine D₃ receptor which limits our ability to exploit it in therapeutic intervention, and which also leads to ambiguous understanding of the origins of clinical effects. For example, in the case of schizophrenia, Slifstein and colleagues conclude in a recent review that two nearly opposite clinical interpretations can be drawn about the role of the D₃ receptor from the currently available data: “either that D₃ receptor blockade is not important for antipsychotic efficacy, or that D₃ represents an underexploited target for antipsychotic therapy that awaits the development of more selective agents”.⁶ For this reason, there remains considerable interest in understanding the function of dopamine receptors, and particularly the D₃ receptor, in such disorders. As such, extensive work has been undertaken to understand and quantify the dopaminergic system using positron emission tomography (PET) imaging.

There are difficulties associated with developing a D₃-selective PET radiotracer. For instance, there is high sequence homology between D₂ and D₃ receptors, primarily at the helical transmembrane section or orthosteric dopamine binding site,⁷ making development of radiotracers that are selective for D₃ over D₂ a challenge. This is evident when considering the most popular ligands in use for dopamine receptor imaging in the clinical setting today. For example, [¹¹C]raclopride and [¹⁸F]fallypride bind to both D₂ and D₃ receptors with comparable affinity.^{8–10} Fortunately, certain areas of the brain only express one type of dopamine receptor,^{11–15} and such radiotracers can still provide useful information in

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these cases. However, numerous other brain areas contain both D₂ and D₃ receptors. When considering D₃ receptors, the issue can be more complicated because, while there are some areas of the brain that predominantly express D₃ receptors (e.g. shell of the nucleus accumbens, islands of Calleja), in other areas D₂ expression is often many-fold higher than D₃ expression.^{11–15} For example, D₂:D₃ ratios have been reported as ~2:1, ~5:1 and ~2:1 in rat, rhesus monkey and human striatal regions, respectively.^{16,17} [¹¹C]-(+)-4-propyl-9-hydroxynaphthoxazine ([¹¹C]PHNO) has been the most clinically utilized tracer to date.^{18,19} However, [¹¹C]PHNO only has ~20-fold selectivity for D₃ over D₂ receptors, and so can only be considered a D₃-preferring radiotracer; this selectivity needs to be accounted for during image analysis.^{6,20}

Developing highly D₃-selective radiotracers that allow quantitation of dopamine D₃ receptors without interference from concurrent binding to D₂ receptors is therefore an active area of research. A number of compound classes have been discovered that, despite the high sequence homology of D₂ and D₃ receptors, have selectivity for D₃ receptors. Many have served as lead scaffolds from which we²¹ and others^{22–39} have attempted to develop D₃-selective PET imaging agents. Unfortunately, many of these tracers are unsuitable for imaging and, to date, there is no radiotracer that has advanced to widespread clinical use. Mach has translated [¹⁸F]fluortripride (FTP, LS-3-134)^{20,24,29} into initial clinical trials; it has picomolar affinity at the D₃ receptor and is ~150-fold selective (vs. D₂), but also binds to 5-HT_{1A} receptors with nanomolar affinity.²⁰ Clinical imaging data has yet to be reported.

A common motif in many D₃ radiotracers is the piperidine or piperazine linker, and desirous of having access to a D₃-selective radiotracer, we have explored developing radiotracers based upon piperazine-containing naphthamide BP897. BP897 is a D₃ partial agonist reported in 1999 by Pilla and co-workers that is moderately selective for D₃ receptors (Table 1).⁴⁰ BP897 and a small series of related analogs were labeled with carbon-11 by Gao and colleagues in 2008, but no imaging data has been reported.³⁴ Efforts have continued to both improve the D₃ selectivity of the scaffold, and develop labeled analogs as PET radiotracers. Another ¹¹C-labeled analog was reported by Turolla *et al.*,³² and although it has ~1200-fold selectivity for D₃ receptors over D₂ receptors and crosses the blood–brain barrier, it does not colocalize with known areas of high D₃ expression (e.g. nucleus accumbens). ¹⁸F-Labeled versions have also been reported by Prante and colleagues,^{30,33} including related phenylazo analogs.^{37,38,41}

For a variety of reasons, the existing radiotracers based upon BP897 have not seen extensive evaluation that includes kinetic modeling. Such reasons include apparent off-target binding either due to poor selectivity for D₃ over D₂ (as well as other receptors such as σ₁) or non-specific binding stemming from high lipophilicity. Alternatively, poor uptake in D₃-rich areas in the brain could also be attributable to occupation of the low levels of D₃ receptors by physiological dopamine.^{22,42,43} This issue could complicate all tracers being developed to image D₃ receptors and should be kept in mind.

Given that there is still a need for a D₃ PET radiotracer, and compounds in this class have some of highest D₃/D₂ selectivities reported to date, we remain interested in developing a radiotracer around the BP897 scaffold. Reflecting this, we were particularly interested in a new series of derivatives reported by Capet *et al.* in 2016,⁴⁴ and especially naphthamide **1** (Table 1) because of its high affinity for D₃ receptors, >1400-fold selectivity for D₃ receptors over D₂, and the possibility for both ¹¹C-labeling in the carbonyl position and ¹⁸F-labeling of the CF₃ group.

Results and discussion

For preliminary evaluation, we elected to prepare [¹¹C]**1** using carbon-fixation common in our laboratory.⁴⁵ Initially unlabeled reference standard **1** was prepared through adaptation of published procedures (Scheme 1).⁴⁴ Briefly, 1-(3-(trifluoromethyl)phenyl)piperazine (**2**) was treated with 4-bromobutyronitrile to give nitrile **3**. Reduction of the nitrile group with borane in THF generated amine **4**, which could be coupled with 2-naphthoyl chloride to provide reference standard **1**.

We were encouraged by the excellent selectivity of **1** reported by Capet using membrane suspensions prepared from stable human D₃ transfected CHO and stable human D₂ transfected HEK293 cells (data shown in Table 1).⁴⁴ However, it is well known that binding affinity values can shift, often by orders of magnitude, when experiments are conducted using *ex vivo* tissue samples instead of purified cells because the latter employs a non-native environment that does not account for target physiology, pathology and micro-environment of the tissue. With compound **1** in hand, we therefore wanted to confirm its selectivity for D₃ over D₂ receptors using rodent brain samples. We conducted autoradiography with rat brain slices using commercially available [³H]R-(+)-7-OH-DPAT (D₃ agonist, D₃ K_i = 0.7 nM, D₂/D₃ = 100 (ref. 46)) and [³H]spiperone (D₂-preferring antagonist, D₂ K_i = 0.08 nM, D₃/D₂ = 5 (ref. 47)).

The K_i and B_{max} values of **1** for the D₃ and D₂ receptors in rat brain slices were determined and are summarized in Table 2. The ratio of D₂:D₃ receptors was 2.7:1, consistent with prior literature reports.¹⁶ However, while we were encouraged to observe that the low nanomolar affinity for the D₃ receptor translated to *ex vivo* tissue samples, our results only demonstrated 14-fold selectivity of **1** for D₃ receptors over D₂ and we were somewhat disappointed that the D₃

Table 1 Lead scaffolds for developing D₃-selective radiotracers

Compound	R	D ₂ K _i (nM)	D ₃ K _i (nM)	D ₂ /D ₃	Ref.
BP897	<i>o</i> -OCH ₃	61	0.92	66	40
1	<i>m</i> -CF ₃	1553	1.1	1400	44

Scheme 1 Synthesis of reference standard **1**. Reagents and conditions: (i) 4-bromobutyronitrile, K_2CO_3 , cat. KI, MeCN, reflux, o/n (99%); (ii) BH_3 , THF, 24–48 h (72%); (iii) 2-naphthoyl chloride, TEA, DCM, 1–2 h (87%).

selectivity dropped considerably when conducting binding affinity experiments in true tissue samples. The 14-fold selectivity for D_3 receptors over D_2 likely makes **1** a D_3 -preferring ligand, similar to PHNO, rather than a D_3 -selective ligand.

The findings are reflected in the phosphorescence images of the rat brain slices (Fig. 1). Thus, baseline images indicating total binding of [3H]R-(+)-7-OH-DPAT revealed the distribution of D_3 (panel A) receptors. As expected, [3H]R-(+)-7-OH-DPAT binding was highest in the nucleus accumbens (ACC), olfactory tubercle (OLFTU) and caudate putamen (CPu), areas known to be rich in D_3 receptors and consistent with the known distribution of [3H]R-(+)-7-OH-DPAT.^{13,46} Densitometrical analysis revealed incubating with 10 μM **1** displaced 50% of [3H]R-(+)-7-OH-DPAT binding (panel B). Non-specific binding of [3H]R-(+)-7-OH-DPAT was identified by incubating with pramipexole (panel C), a known D_3 -preferring ligand (D_3 K_i = 0.37 nM, D_2/D_3 = 19 (ref. 49)). Notably, non-specific binding was found to be 50% of the total signal, suggesting that 10 μM **1** displaced all specifically bound [3H]R-(+)-7-OH-DPAT, and that the remaining signal was due to non-specific binding.⁴⁸

Reflecting the colocalization of D_2 and D_3 receptors in the rat brain, the higher expression of D_2 receptors compared to D_3 , and that while [3H]spiperone is D_2 -preferring it also binds to D_2 and 5-HT $_2$ receptors, total binding of [3H]spiperone was apparent in the same areas as [3H]R-(+)-7-OH-DPAT but at levels ~ 3 times higher (panel D). There was increased binding of [3H]spiperone in the cortical regions, consistent with the higher levels of D_2 receptors in the cortex compared to D_3 ,¹³ as well as possible off target binding to 5-HT $_2$ receptors.⁵¹ Densitometrical analysis revealed incubation with 10 μM **1** displaced 15% of [3H]spiperone binding (panel E), likely corresponding to ligand specifically bound to D_3 receptors. Both [3H]spiperone specific binding to D_2 receptors and non-specific binding was identified by co-incubating with 10 μM of the D_2 -preferring antagonist butaclamol (D_2 K_i = 1.0 nM, D_3/D_2 = 5 (ref. 47)). This displaced 55% of the [3H]spiperone signal, suggesting that 45% of the signal corresponded to non-specifically bound ligand (panel F).

Fig. 1 Rat brain autoradiography studies (CPu: caudate putamen, ACC: nucleus accumbens, OLFTU: olfactory tubercle). Rat brain atlas figure is reprinted from the rat brain in stereotaxic coordinates. Copyright (1998), with permission from Elsevier.⁵⁰

We next investigated the radiosynthesis of [^{11}C]**1** (Scheme 2). [^{11}C]CO $_2$ (3 Ci) was prepared in a GE PETTrace cyclotron, and bubbled into freshly prepared Grignard reagent **5**. The reaction was quenched with thionyl chloride and catalytic DMF to generate [^{11}C]2-naphthoyl chloride **6**, which was reacted with amine **4** to generate [^{11}C]**1**. Purification by semi-preparative HPLC and reformulation provided [^{11}C]**1** in low radiochemical yield (2 ± 1 mCi, 0.1% non-corrected RCY based upon 3.0 Ci of [^{11}C]CO $_2$, n = 4), but generated enough radiotracer for pre-clinical PET imaging studies. The product was obtained in good radiochemical purity (RCP) (>95%), high specific activity (>2000 Ci mmol $^{-1}$) and quality control testing revealed doses were suitable for use in rodents. Formulated [^{11}C]**1** was stable for at least 2 h after end-of-synthesis. *In vitro* stability in rat and human blood was also examined by adding [^{11}C]**1** to ~ 1 mL samples of each. After 30 min, plasma was separated by centrifugation and, in each case, $\sim 60\%$ of the radioactivity remained in the plasma. The plasma samples were analyzed by radio-thin layer chromatography (radio-TLC), and revealed >95% intact radiotracer.

Finally, [^{11}C]**1** was employed in preliminary baseline rodent PET imaging studies to evaluate *in vivo* behavior and imaging properties. The radiotracer (331 ± 37 μCi) was administered to Sprague–Dawley rats *via* tail vein injection (n = 2), and imaging was conducted for 60 min. Images were reconstructed and frames were summed and smoothed. Regions-of-interest (ROIs) were drawn on multiple planes for areas of interest, and the volumetric ROIs were then applied to the full dynamic data set to generate time–radioactivity curves (TACs) (Fig. 2).

Table 2 *Ex vivo* autoradiography data⁴⁸

Compound	K_i D_2 (nM)	B_{max} D_2 (nM)	K_i D_3 (nM)	B_{max} D_3 (nM)	D_2/D_3
1	647 ± 79	31 ± 1.85	46.5 ± 10.7	11.5 ± 0.75	14

Scheme 2 Radiosynthesis and analytical HPLC trace of $[^{11}\text{C}]1$. Reagents and conditions: (i) $[^{11}\text{C}]\text{CO}_2$; (ii) SOCl_2 , THF, cat. DMF; (iii) **4**, TEA, THF, rt, 5 min; * = ^{11}C .

We were encouraged to see good CNS penetration of the radiotracer, as well as increased retention in identifiable brain regions. Notably, highest uptake was in the olfactory tubercle (peak SUV ~ 1.5), caudate putamen (including nuclear accumbens) region (peak SUV ~ 1.2) and hypothalamus (peak SUV ~ 0.9), known D_3 -rich areas of the brain^{1,13} that are consistent with the highest distribution of D_3 receptors identified in the $[^3\text{H}]\text{R}(-)-7\text{-OH-DPAT}$ autoradiography experiments described above. There was also uptake in the cerebellum (Cbm) that was lower (peak SUV ~ 1.0), and is also consistent with the known distribution of D_3 receptors in the rat brain. D_3 receptors are found in the cerebellum, but at lower areas than, for example, the caudate putamen and olfactory tubercle.^{1,10} There was also uptake of $[^{11}\text{C}]1$ in both the thyroid (Thy) and pituitary (Pit) glands, consistent with other radiotracers based upon the BP897 scaffold,^{32,41} and possibly indicative of non-specific binding.¹³

Finally, in one of the rodent imaging studies, a blood sample was removed 15 min post-injection for metabolite analysis and, although the signal was low, radio-HPLC analysis revealed $\geq 80\%$ intact $[^{11}\text{C}]1$ in the plasma fraction.⁵²

Experimental

Chemistry

General considerations. All solvents and reagents were commercially available and used without further purification

unless otherwise stated. NMR spectra were recorded with a Varian 400 MHz instrument at room temperature with tetramethylsilane (TMS) as an internal standard. Mass spectra were performed on an Agilent Q-TOF HPLC-MS or VG (Micro-mass) 70-250-S Magnetic sector mass spectrometer employing the electrospray ionization (ESI) method. HPLC was performed using a Shimadzu LC-2010A HT system. All procedures including anhydrous solvents were performed with rigorously dried glassware under inert atmosphere.

Synthesis of reference standard 1

4-(4-(3-(Trifluoromethyl)phenyl)piperazin-1-yl)butanenitrile (3). 1-(3-Trifluoromethyl)phenylpiperazine (0.3 g, 1.3 mmol) in K_2CO_3 (0.198 g, 1.43 mmol), cat. KI (1 or 2 crystals), and anhyd. MeCN (6.5 mL) were stirred for 5 min at room temperature (rt). 4-Bromobutyronitrile (0.215 mg, 1.43 mmol) was added, the flask was sealed and the reaction was heated to reflux for 14 h. The reaction was cooled, quenched with water (4 mL), and the product was extracted with CH_2Cl_2 (3×15 mL), and dried (Na_2SO_4) to give title compound **3** (0.42 g, 99%). The product was characterized and used directly in the next step. Rf 0.76 (20% MeOH/EtOAc). HRMS (ESI) m/z $[\text{M}^+]$ calcd for $\text{C}_{15}\text{H}_{18}\text{F}_3\text{N}_3$: 297.1526, found: 298.1531 ($\text{M} + \text{H}$). ^1H NMR (400 MHz, chloroform- d) δ 7.32 (t, $J = 8.0$ Hz, 1H), 7.09 (d, $J = 2.0$ Hz, 1H), 7.07–7.01 (m, 2H), 3.23–3.17 (m, 4H), 2.59–2.53 (m, 4H), 2.48 (t, $J = 6.7$ Hz, 2H), 2.42 (t, $J = 7.1$ Hz, 2H), 1.83 (q, $J = 6.9$ Hz, 2H). ^{13}C NMR (101 MHz, chloroform- d) δ 151.31, 131.41, 129.76, 119.80, 118.83, 115.88, 112.18, 56.12, 52.80, 48.54, 22.64, 14.83. ^{19}F NMR (376 MHz, chloroform- d) δ -62.59.

4-(4-(3-(Trifluoromethyl)phenyl)piperazin-1-yl)butan-1-amine (4). To a mixture of nitrile **3** (0.386 g, 1.3 mmol) in anhydrous THF (10 mL) at 0°C , under argon, was slowly added borane-THF (7.8 mmol, 1 M in THF) over 5 min. The reaction allowed to warm to rt o/n and stirred for a total of 48 h. The mixture was cooled to 0°C and quenched (MeOH). The product was suspended in 6 M HCl/MeOH, stirred for 2 h at rt and then concentrated. The pH was adjusted to alkaline with $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$, and the reaction mixture was extracted (EtOAc) and dried (Na_2SO_4). The product was purified by silica gel flash chromatography (20% MeOH/80% EtOAc) to give amine **4** (0.355 g, 72%). Rf = 0.07 (20% MeOH/EtOAc). HRMS (ESI) m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{15}\text{H}_{22}\text{F}_3\text{N}_3$: 301.1841, found: 302.1841 ($\text{M} + \text{H}$). ^1H NMR (400 MHz, chloroform- d) δ 7.24 (d, $J = 15.7$ Hz, 1H), 7.03 (s, 1H), 6.97 (t, $J = 7.2$ Hz, 2H), 3.44 (s, 2H), 3.14 (q, $J = 8.5, 6.7$ Hz, 4H), 2.67 (t, $J = 6.5$ Hz, 2H), 2.50 (q, $J = 7.0, 6.0$ Hz, 4H), 2.31 (q, $J = 6.0, 5.4$ Hz, 2H), 1.47 (th, $J = 6.7, 3.4$ Hz, 4H). ^{13}C NMR (101 MHz, chloroform- d) δ 151.28, 131.07, 129.55, 125.65, 118.52, 115.51, 111.97, 58.20, 52.91, 48.47, 41.66, 30.50, 24.07. ^{19}F NMR (376 MHz, chloroform- d) δ -62.68.

N-(4-(4-(3-(Trifluoromethyl)phenyl)piperazin-1-yl)butyl)-2-naphthamide (1). In a dry round bottom flask was added amine **4** (0.15 g, 0.5 mmol), anhydrous CH_2Cl_2 (1 mL), Et_3N (0.253 g, 2.5 mmol) and the mixture was stirred for 5 min. To this was added 2-naphthoyl chloride (0.114 g, 0.6 mmol). The reaction was stirred at rt for 2 h, then extracted with CH_2Cl_2

Fig. 2 Summed sagittal rodent image (0–60 min post-injection of $[^{11}\text{C}]1$) and TACs for $[^{11}\text{C}]1$ (Cbm: cerebellum, CPu/Acc: caudate putamen/nucleus accumbens, OLFTU: olfactory tubercle).

(3 × 15 mL), dried (Na₂SO₄) and purified by silica gel flash chromatography (20% MeOH/EtOAc) to give product **1** (0.197 g; 87%). *R_f* = 0.4 (20% MeOH/EtOAc). HRMS (ESI) *m/z* [M + H]⁺ calcd for C₂₆H₂₈F₃N₃O: 456.2257, found: 456.2261. ¹H NMR (400 MHz, chloroform-*d*) δ 8.27 (d, *J* = 1.6 Hz, 1H), 7.85–7.79 (m, 4H), 7.52 (ddd, *J* = 8.1, 6.8, 1.4 Hz, 1H), 7.46 (ddd, *J* = 8.2, 6.9, 1.3 Hz, 1H), 7.32 (t, *J* = 7.9 Hz, 1H), 7.09–6.96 (m, 4H), 3.54 (q, *J* = 6.1 Hz, 2H), 3.21 (dd, *J* = 6.4, 3.8 Hz, 4H), 2.64 (dd, *J* = 6.2, 3.9 Hz, 4H), 2.51 (t, *J* = 6.8 Hz, 2H), 1.72 (dd, *J* = 6.3, 3.3 Hz, 4H). ¹³C NMR (101 MHz, chloroform-*d*) δ 167.84, 151.10, 134.60, 132.54, 132.12, 129.63, 128.84, 128.37, 127.65, 127.28, 126.59, 123.72, 118.71, 116.02, 112.21, 57.64, 52.72, 48.19, 39.82, 27.29, 23.93. ¹⁹F NMR (376 MHz, chloroform-*d*) δ –62.68.

Radiochemistry

General considerations. Standard reagents and solvents were commercially available and used without further purification, unless otherwise noted: sodium chloride, 0.9% USP and sterile water for injection, USP were purchased from Hospira; dehydrated alcohol for injection, USP was obtained from Akorn Inc.; molecular sieves were purchased from Alltech; HPLC columns were acquired from Phenomenex. Other synthesis components were obtained as follows: sterile filters were acquired from Millipore; C18 Sep-Paks were purchased from Waters Corporation; 10 cc sterile vials were obtained from HollisterStier. Sep-Paks were flushed with 10 mL of ethanol followed by 10 mL of sterile water prior to use.

Radiosynthesis of [¹¹C]1**.** [¹¹C]CO₂ was produced with a GE PETTrace cyclotron *via* the ¹⁴N(p,α)¹¹C reaction. High purity N₂ (g) containing 0.5% O₂ was irradiated to generate [¹¹C]CO₂ (~3 Ci), which was delivered to a GE TRACERLab FXC-Pro synthesis module and bubbled into a solution of Grignard reagent **5** (100 μL 0.5 M in 100 μL THF) for 5.5 min at 7 mL min^{–1}. This was followed by addition of thionyl chloride (12 μL) with cat. DMF (1 drop) in THF (200 μL) for 1.5–2 min to generate **6**. A solution of amine **4** (3.5–4 mg) and Et₃N (115 μL) in THF (200 μL) was then added and the reaction was stirred for 5 min at rt. The crude reaction mixture was diluted with 0.6 mL of HPLC mobile phase, passed through a Gelman Acrodisc 13 CR PTFE 0.2 μm filter, and purified by semi-preparative HPLC (column: Luna 10 μ C18 100A (250 × 10 mm) mobile phase: 60% MeCN, 20 mM NH₄OAc, 2 mL HOAc, flow rate: 2.5 mL min^{–1}). The desired peak was collected (*t_R* ~ 10 min, see Fig. 3 for a representative semi-preparative radio-HPLC trace). The collected HPLC fraction was diluted in water (50 mL water with 1 mL 1% NH₄OH) and passed through a C18 sep-pak (waters 1 cc) to trap [¹¹C]**1**. The cartridge was rinsed with further water (2 mL) to remove residual HPLC mobile phase. The product was eluted with ethanol (0.2 mL) and diluted with saline (1.8 mL) such that the final product was formulated in 2 mL of 10% ethanolic saline with a pH of 5.5. Doses were passed through a 0.22 μm sterile filter into a sterile vial prior to use in pre-clinical studies.

Quality control of [¹¹C]1**.** Doses produced were assessed *via* standard quality control techniques and were appropriate for rodent studies. Radiochemical purity of products was assessed using Shimadzu LC-2010A HT system equipped with UV-vis and Bioscan B-FC-1000 radiation detectors (column: Luna 10 μ C18 100A (150 × 4.6 mm); mobile phase: 40% MeCN: 20 mM NH₄OAc, 2 mL HOAc, flow rate: 2.0 mL min^{–1}; *t_R* ~ 7.5 min, see Scheme 2 for a typical radio-HPLC trace). Dose pH was analyzed using colorpHast® pH 2.0–9.0 nonbleeding pH-indicator strips, and needed to be between 4.5 and 7.5 for *in vivo* studies.

Autoradiography

D₃ receptor saturation studies. Tissue sections were preincubated for 5 min in PBS, pH 7.4 at 25 °C or 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 30 mM EDTA at rt. The slides for total binding were then incubated in 5–60 nM [³H]R-(+)-7-OH-DPAT (American Radiochemical, Inc) in the above buffer for 15 minutes at rt. Pramipexole (10 μM) was added to an adjacent slide to define non-specific binding. Sections were washed twice for a total of 2 min in ice cold buffer then rinsed briefly in distilled water. Sections were air-dried before exposing to tritium-sensitive phosphoimaging screen for 3–5 days.

D₃ receptor displacement studies. Tissue sections were preincubated for 5 min in PBS, pH 7.4 at 25 °C or 50 mM Tris-HCl, pH 8.0 (±150 mM NaCl) at rt. Slides were then incubated in 35 nM [³H]R-(+)-7-OH-DPAT (American Radiochemical, Inc) with no addition (total binding) or various concentrations (300 nM to 1 mM, *f.c.*) of compound **1** in the above buffer for 15 min at rt. Pramipexole (10 μM) was added to an adjacent slide to define non-specific binding. Sections were washed and exposed to a tritium-sensitive phosphoimaging screen as described above.

D₂ receptor saturation studies. Tissue sections were preincubated for 5 min in PBS, pH 7.4 at 25 °C or 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 30 mM EDTA at room temperature. The slides for total binding were then incubated in 0.5–50 nM [³H]spiperone (American Radiochemical, Inc) in the above buffer for 30 min at rt. (+)Butaclamol (10 μM) was

Fig. 3 Semi-preparative HPLC trace for purification of [¹¹C]**1**.

added to an adjacent slide to define non-specific binding. Sections were washed, dried and exposed to a phosphoimaging screen as described above.

D₂ receptor displacement studies. Tissue sections were preincubated for 5 min in PBS, pH 7.4 at 25 °C or 50 mM Tris-HCl, pH 8.0 ($\pm$ 150 mM NaCl) at rt. Slides were then incubated in 5 nM [³H]spiperone (American Radiochemical, Inc) with no addition (total binding) or various concentrations (300 nM to 1 mM, f.c.) of compound 1 in the above buffer for 30 minutes at room temperature. (+)Butaclamol (10 μ M) was added to an adjacent slide to define non-specific binding. Sections were washed, dried and exposed to a phosphoimaging screen as described above.

Densitometry analysis

Calibrated plastic radioactive standards were included with tissue sections to correct for variations in exposure. After development (Fuji Typhoon FLA 7000), images were analyzed with ImageQuant software (Fuji). Phosphoimager units were converted to fmol on the basis of image densities overlaying the radioactive standards and accounting for the specific activity of the radiotracer.

Rodent PET imaging

Rodent PET imaging studies were conducted using a Concorde Microsystems P4 PET scanner. Anesthesia was induced in healthy, female Sprague–Dawley rats (320 $\pm$ 37 g, n = 2) using isoflurane/O₂, and anesthesia was maintained throughout imaging. Body temperature was maintained by a heating pad. A transmission scan for attenuation correction was acquired prior to administration of [¹¹C]1 (331 $\pm$ 37 μ Ci) *via* tail vein injection. Imaging was conducted for 60 min. Following the scan, emission data were corrected for decay, dead time and random coincidences, before reconstruction using an iterative ordered subset expectation maximization (3D maximum *a posteriori*, 3D-MAP) method to generate the reconstructed images. Frames were summed and smoothed, and ROIs were drawn on multiple planes for each area of interest. The volumetric ROIs were applied to the full dynamic data sets to generate time-radioactivity curves. Standardized uptake values (SUVs) were calculated from time-radioactivity data.

In vitro stability

[¹¹C]1 (50 $\pm$ 25 μ Ci) was added to $\sim$ 1 mL of rat or human blood. After 10 min, the samples were centrifuged at 7500 RPM for 5 min, and plasma was separated. In each case, $\sim$ 60% of the radioactivity remained in the plasma. Radioactivity in the plasma samples was analyzed by radio-TLC. Following development, plates were exposed to a phosphoimaging screen and analyzed by the Fuji Typhoon FLA 7000.

Metabolite analysis

During microPET imaging, blood was drawn from the tail vein of one rat 15 min post-injection of the radiotracer.

The sample was centrifuged at 7500 RPM for 5 min, and plasma was separated. Radioactivity in the plasma sample was analyzed by radio-HPLC and revealed $\geq$ 80% intact radiotracer.

Conclusions

In summary, a potential new D₃-selective radiotracer ([¹¹C]1) has been synthesized and evaluated *ex vivo* and *in vivo*. [¹¹C]1 exhibited specific binding to D₃ receptors and 14-fold selectivity over D₂ receptors using *ex vivo* autoradiography experiments with rat brain. Selectivity is lower than the 1400-fold reported previously in literature cell studies. Rodent PET imaging revealed reasonable uptake of the radiotracer in areas of the brain known to be rich in D₃ receptors. Future experiments aimed at understanding the *in vivo* selectivity for D₃ receptors, identifying non-specific binding, and investigating the sensitivity of the radiotracer to endogenous dopamine will occur following scale up and optimization of the radiosynthesis, and translation of the radiotracer into nonhuman primates, where small D₃-rich areas of the brain are more accurately identifiable and quantifiable.

Ethics statement

All animal experiments were conducted under the supervision of the University of Michigan Institutional Animal Care and Use Committee (IACUC) and were conducted according to approved protocols in accordance with all applicable federal, state, local and institutional laws or guidelines governing animal research.

Conflicts of interest

The authors declare no conflicts of interest.

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