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[³H]4-(Dimethylamino)-*N*-(4-(4-(2-Methoxyphenyl)Piperazin-1-yl)-Butyl)Benzamide: A Selective Radioligand for Dopamine D₃ receptors. II. Quantitative Analysis of Dopamine D₃ and D₂ Receptor Density Ratio in the Caudate-Putamen

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Abstract

4-(Dimethylamino)-*N*-(4-(4-(2-methoxyphenyl)piperazin-1-yl)butyl)-benzamide (WC-10), a *N*-phenyl piperazine analog, displays high affinity and moderate selectivity for dopamine D₃ receptors versus dopamine D₂ receptors (Chu et al. [2005] *Bioorg Med Chem* 13:77–87). In this study, WC-10 was radiolabeled with tritium (specific activity = 80 Ci/mmol), and quantitative autoradiography studies were conducted using rhesus monkey and Sprague-Dawley rat brain sections. *K_d* values for the binding of [³H]WC-10 to D₃ receptors obtained from quantitative autoradiography with rhesus monkey and rat brain sections are in agreement with *K_d* values obtained from cloned human and rat receptors (Xu et al. [2009] *Synapse* 63:717–728). The D₂ selective antagonist [³H]raclopride binds with 11-fold higher affinity to human HEK D_{2L} (*K_d* = 1.6 nM) than HEK D₃ (*K_d* = 18 nM) receptors; [³H]raclopride binds to rat Sf9 rD_{2L} receptors with a *K_d* of 6.79 nM, a value that is 4-fold lower than binding to human HEK D_{2L} receptors and 2.5-fold higher than binding to rat Sf9 rD₃ receptors. In vitro quantitative autoradiography studies with [³H]WC-10 and [³H]raclopride were conducted on adult rat and rhesus monkey brain sections. A mathematical model for calculating the absolute densities of dopamine D₂ and D₃ receptors based on the in vitro receptor binding data of [³H]WC-10 and [³H]raclopride was developed.

Keywords

dopamine; DA D₃/D₂ receptors; quantitative autoradiography

INTRODUCTION

D₁-like and D₂-like receptor subtypes are the two major classes of dopamine receptors. The D₁-like receptor subtypes include the D₁ (D_{1a}) and D₅ (D_{1b}) receptors and stimulation of D₁-like receptors activates adenylate cyclase. The D₂-like receptor subtypes consist of the D₂ (both long and short isoforms), D₃, and D₄ receptors. Agonist stimulation of D₂-like receptors (a) inhibits adenylate cyclase activity and (b) increases the release of arachadonic acid and phosphatidylinositol hydrolysis (Luedtke and Mach, 2003; Neve et al., 2004).

D₂-like receptors exist in two interconvertible affinity states for their natural agonist dopamine: a high-affinity state and a low-affinity state (Sibley et al., 1982). Under physiological conditions, dopamine binds predominantly to the high affinity state and mediates the activation of the second-messenger cascade. Although autoradiography studies using the D₂/D₃ agonists, [³H]7-OH-DPAT and [³H]quinpirole, under conditions minimizing binding to the D₂ receptor, suggest that dopamine D₃ receptors are localized in the ventral striatum and the Islands of Calleja (Gehlert et al., 1992; Kaichi et al., 2000; Levesque et al., 1992), there are data indicating that the density of dopamine D₃ receptors measured with agonists [³H]7-OH-DPAT and [³H]PD128907 is higher in the adult rat caudate-putamen than in the islands of Calleja (Hillefors and von Euler, 2001; Hillefors et al., 1999). The high affinity state is believed to be the functionally important state (George et al., 1985; Leff, 1995). Nevertheless, the low affinity state of D₂-like receptors, and its conversion to a high affinity state, needs to be further investigated in evaluating their regulatory functions in both diseased and healthy individuals (Briand et al., 2008; Graff-Guerrero et al., 2009; King et al., 2009; Skinbjerg et al., 2009).

It has been difficult to obtain ligands specifically selective for D₂ or D₃ receptors because of the high degree of amino acid homology in the helical transmembrane spanning regions of those receptors. Some selective dopamine D₃ agonists (7-OH-DPAT and PD128947) and dopamine D₂ agonists (PHNO) are available, but selective D₂ or D₃ receptor antagonists are not well documented (Ginovart et al., 2006; Luedtke and Mach, 2003; Vasdev et al., 2007). The development of high affinity D₃ and D₂ receptor antagonists would be valuable for studying the regulating mechanism of these receptors in neurological disorders.

A *N*-phenyl piperazine analog, 4-(dimethylamino)-*N*-(4-(4-(2-methoxyphenyl)piperazin-1-yl)butyl)benzamide (WC-10), displays higher affinity and binding selectivity for dopamine D₃ receptors versus dopamine D₂ receptors (Chu et al., 2005). In a previous publication, WC-10 was radiolabeled with tritium (specific activity = 80 Ci/mmol) and the binding of [³H]WC-10 to genetically cloned human and rat dopamine D_{2L} and D₃ receptors was evaluated in vitro (Xu et al., 2009). In-vitro autoradiography studies showed that [³H]WC-10 specifically labeled the D₃ sites of striatum in adult Sprague-Dawley rat and rhesus monkey brains. In this article, saturation binding with [³H]WC-10 was carried out to evaluate the binding affinity (*K_d*) with quantitative autoradiography. Comparatively, we characterized D₂ selective antagonist [³H]raclopride binding properties at D₂ or D₃ receptors. In vitro quantitative autoradiography studies combining the D₃/D₂ ligand, [³H]WC-10 and the D₂/D₃ ligand, [³H]raclopride, were also conducted on adult rat and rhesus monkey brain sections. A mathematical model for calculating the absolute densities of dopamine D₂ and D₃ receptors based on the in vitro binding data obtained from

[³H]WC-10 and [³H]raclopride was also developed and used to determine the density of D₂ and D₃ dopamine receptors.

MATERIALS AND METHODS

Precursor synthesis and radiolabeling

[³H]WC-10 (Fig. 1) was synthesized by American Radiolabeled Chemicals (St. Louis, MO) by alkylation of the des-methyl precursor with [³H]methyl iodide. The specific activity of the radioligand was 80 Ci/mmol. The detailed synthesis scheme for [³H]WC-10 was reported (Xu et al., 2009).

Drugs and preparation

Chemical reagents and the standard compounds were purchased from Sigma (St. Louis, MO) and Tocris (Ellisville, MO). [³H]raclopride (60.1 Ci/mmol) was purchased from Perkin Elmer Life Sciences (Boston, MA).

Membrane homogenate preparation

HEK or Sf9 cells expressing human or rat D₂ and D₃ receptors were harvested by centrifugation at 6000g for 10 min (Luedtke et al., 2000). The cell pellet was resuspended in cold (4°C) homogenization buffer (50 mM Hepes, pH 7.4, 0.1 mM EDTA, 1 mM DTT) by vigorous vortexing and then homogenized using an Ultra-Turrax T8 polythion homogenizer (IKA Works, Inc, Wilmington, NC). The homogenate was centrifuged at 40,000g for 10 min at 4°C and the resulting crude membrane pellet was resuspended in homogenization buffer. Aliquots were stored at -80°C until used. The protein concentration of the suspension was determined using the DC protein assay (Bio-Rad, Hercules, CA) and averaged 1–2 mg of protein/ml of stock solution.

Radioligand binding assay

Scatchard analysis of [³H]raclopride binding to receptor membrane

homogenates—Membrane homogenates were diluted and incubated for 60 min with the radioligand [³H]raclopride in a total volume of 150 µl at 25°C in 96-well polypropylene plates (Fisher Scientific, Pittsburgh, PA). The amount of protein added to each well was 10–20 µg for genetically cloned D_{2L} and D₃ receptors. The concentrations of the radioligand ranged from 0.1 to 30.0 nM, reactions were terminated by the addition of 150 µl of cold wash buffer (10 mM Tris-HCl, 150 mM NaCl, pH 7.4, at 4°C) using a 96-channel transfer pipette (Fisher Scientific, Pittsburgh, PA), and the samples were harvested and filtered rapidly to 96-well fiberglass filter plate (Millipore, Billerica, MA) that had been presoaked with 100 µl of 50 mM Tris-HCl buffer, pH 8.0 for 1 h. Each filter was washed with 200 µl of ice-cold wash buffer for a total of three washes and 150 µl of scintillation fluid was added in each well. A Wallac 1450 MicroBeta liquid scintillation counter (Perkin Elmer, Boston, MA) was used to quantitate the bound radioactivity. Nonspecific binding was determined from samples which contained 10 µM haloperidol.

The equilibrium dissociation constant (K_d) and maximum number of binding sites (B_{max}) were determined by a linear regression analysis of the transformed data using the method of Scatchard (1949).

Data from saturation radioligand binding studies were transformed to determine the Hill coefficient, n_H , defined as:

$$\log \frac{B_s}{B_{\max} - B_s} = -\log K_d + n_H \log L \quad (1)$$

(Hill, 1910; McGonigle and Molinoff, 1989). B_s is the specific radioligand binding, L is the concentration of radioligand. n_H , Hill slope, was determined from a Hill plot of

$$\log \frac{B_s}{B_{\max} - B_s} \text{ vs. } \log L.$$

Scatchard analysis of autoradiography binding assay with [³H]WC-10 to rhesus monkey and rat brain sections—Rhesus monkey and rat brain adjacent sections were incubated with [³H]WC-10, with concentrations ranging from 0.5 to 16.0 nM. Quantitative imaging analysis for the anatomical regions of interest (ROI), caudate and putamen, was performed using a Beta Imager 2000Z Digital Beta Imaging System. Scatchard binding analysis was carried out as described in the previous section. Nonspecific bond activity at each concentration was defined from slides using 1 μM S(–)-Eticlopride.

Quantitative autoradiography

Intact brains from ~500 g male Sprague-Dawley rats at age of 5 months and a 5 kg male rhesus monkey at age of 6 years (euthanized due to a pancreatic tumor) were flash frozen in dry ice, precooled in isopentane, and stored at –80°C until used. Coronal sections (20 μm) were cut with a Microm cryotome and mounted on superfrost plus glass slides (Fisher Scientific, Pittsburgh, PA). Sections were incubated with 4 nM of [³H]WC-10 or 10 nM of [³H]raclopride in the same buffer used for binding assays, in the presence or absence of 1 μM cold S(–)-Eticlopride (to define nonspecific binding) for 30 min, and then rinsed five times at 1 min intervals each time with ice-cold buffer. Slides were incubated in the wide open staining jar, and the free radioligand concentration loss was less than 5% after ligands bound to brain sections. Slides were dried and made conductive by coating the free side with a copper foil tape. Slides were then placed in the gas chamber [mixture of argon and triethylamine (Sigma-Aldrich, USA)] of a gaseous detector system, the Beta Imager 2000Z Digital Beta Imaging System (Biospace, France). After the gas was well mixed and a homogenous state was reached, further exposure for 24 h yielded high-quality images. [³H]Microscale (American Radiolabeled Chemicals, St. Louis, MO) was counted at the same time as a reference for total radioactivity quantitative analysis. Quantitative analysis was performed with the program β-Vision Plus (BioSpace, France) for the anatomical ROI.

In comparison with digital Beta Imager, the dried slides with brains sections were also exposed to a ³H-sensitive film (³H-Hyperfilm, Amersham, Buckinghamshire, UK) for 1 or 3 months, and the film was developed with a Kodak X-OMAT 2000A processor.

Determination of absolute densities ratio of D₂ and D₃ receptors from autoradiography studies—[³H]WC-10 and [³H]raclopride bind to dopamine D₂ and D₃ receptors with different labeling proportions. On the basis of receptor homogenates binding studies, 4 nM [³H]WC-10 will label 2% of D₂ receptors and 50% of D₃ receptors in rat striatum, and 5% of D₂ receptors and 78% of D₃ receptors in monkey striatum. These values can be derived from the saturation binding isotherm (i.e., Michaelis–Menten equation):

$$\frac{B}{B_{\max}} = \frac{L}{L + K_d}, \quad (2)$$

where B is the specific receptor bound radioligand, $B_{\max}$ is the receptor density, L is the radioligand concentration, and K_d is the equilibrium dissociation constant. In this study, the L value was 4 nM and K_d values were 1.16 nM for D₃ and 76 nM for D_{2L} receptors in the

monkey study, which were determined using binding data with cloned human receptors (Table I). In contrast, K_d values were 3.94 nM for D_3 and 158 nM for D_2 receptors in the rat brain study, which were determined from binding study with cloned rat receptors (Table I). Using the same analysis and binding data (Table I), 10 nM [3 H]raclopride will label (a) 60% of D_2 receptors and 37% of D_3 receptors in rat striatum, and (b) 86% of D_2 receptors and 36% of D_3 receptors in monkey striatum.

The total bound amount of receptors of 4.0 nM [3 H]WC-10 or 10 nM [3 H]raclopride binding can be expressed by the formulas:

$$[^3\text{H}] \text{WC} - 10: \quad a_1 D_2 + b_1 D_3 = B_1 \quad (3)$$

$$[^3\text{H}] \text{raclopride:} \quad a_2 D_2 + b_2 D_3 = B_2, \quad (4)$$

Where a_1 and b_1 are the fractional occupancies of 4 nM of [3 H]WC-10 to D_2 and D_3 receptors; B_1 is the apparent receptor binding density ($D_2 + D_3$) directly measured from autoradiography studies of 4.0 nM of [3 H]WC-10; a_2 , b_2 , and B_2 are the same parameters for 10 nM of [3 H]raclopride; D_2 , D_3 are the absolute densities of D_2 and D_3 receptors. The absolute densities of D_2 and D_3 receptors were calculated by solving the simultaneous equations:

$$D_2 = \frac{b_2 B_1 - b_1 B_2}{a_1 b_2 - a_2 b_1} \quad (5)$$

$$D_3 = \frac{a_1 B_2 - a_2 B_1}{a_1 b_2 - a_2 b_1} \quad (6)$$

It is assumed that (a) the law of mass actions applies in this study; (b) all receptors are equally accessible to both [3 H]WC-10 and 10 nM [3 H]raclopride; (c) both radioligands are antagonists and label high and low affinity sites of dopamine D_2 or D_3 receptors with equal affinity; (d) neither receptor nor ligand are altered by binding; (e) free radioligand concentration remains unchanged after binding.

RESULTS

Saturation experiments

Saturation experiments with genetically cloned receptors—Direct saturation binding studies were carried out using [3 H]WC-10 and [3 H]raclopride with cloned human hD₃, hD_{2L} receptors on HEK cell membranes and rat rD₃, rD_{2L} receptors on Sf9 cell membranes. Detailed descriptions and data for [3 H]WC-10 binding were reported previously (Xu et al., 2009). The Scatchard plots and K_d values of [3 H]raclopride binding to human hD₃, hD_{2L} receptors expressed in HEK cells (Figs. 2A and 2B) and rat rD₃, rD_{2L} receptors expressed in Sf9 cell membranes (Figs. 2C and 2D) are shown in Figure 2. The K_d values of the receptor-radioligand binding of [3 H]WC-10 and [3 H]raclopride to human and rat D_{2L}, D₃ receptors and their binding selectivity ratios are summarized (Table I). [3 H]WC-10 binds with a 66-fold higher affinity to human HEK D₃ than to HEK D_{2L} receptors, with a dissociation constant (K_d) of 1.2 nM. [3 H]WC-10 binds to rat Sf9 D₃ receptors with a K_d of 3.9 nM, demonstrating a 3-fold lower affinity for human HEK D₃ receptors and a 40-fold higher affinity for rat Sf9 D_{2L} receptors. [3 H]raclopride binds with a 11-fold higher affinity to human HEK D_{2L} (K_d = 1.6 nM) than to HEK D₃ (K_d = 18 nM) receptors; and [3 H]raclopride binds to rat Sf9 rD_{2L} receptors with a K_d of 6.79 nM, a value that is 4-fold

lower than binding to human HEK D_{2L} receptors and 2.5-fold higher than binding to rat Sf9 rD₃ receptors. Because the average n_H values are close to unity, results indicate that receptor binding data fit well to a one site model where the binding is noncooperative.

Saturation experiments with rhesus monkey and rat brain sections—Direct saturation binding studies were conducted using [³H]WC-10 with rhesus monkey caudate (Fig. 3A), putamen (Fig. 3B) and rat caudate-putamen (Fig. 3C). The K_d and n_H values of the receptor-radioligand binding from Scatchard plots of [³H]WC-10 to rhesus monkey and rat caudate-putamen are shown in Figure 3. n_H values were close to equity, which suggest that the binding of [³H]WC-10 is to single binding site. [³H]WC-10 binds with dissociation constants (K_d s) of 1.3 and 1.1 nM to rhesus monkey caudate and putamen respectively. [³H]WC-10 binds to Sprague-Dawley rat striatum with dissociation constant (K_d) of 3.4 nM. Similar results of dissociation constants (K_d s) were also observed in Fisher 344 and Wistar rat brain (data not shown). These K_d values are in good accordance with the K_d values obtained from [³H]WC-10 binding to cloned human or rat receptors (Xu et al., 2009). Therefore, the K_d values obtained from cloned receptors are suitable values for receptor occupancy estimates for the in-vitro autoradiography of this study.

Quantitative autoradiography

Autoradiography showed that [³H]WC-10 and [³H]raclopride labeled the D₂/D₃ sites in monkey striatal caudate and putamen, as well as the D₂/D₃ sites in rat striatum (Figs. 4A–4D). The sensitivity limit of Beta Imager 2000Z Digital Beta Imaging System was 0.07 dpm/mm². A tritium standard [³H]Microscale (Fig. 4E) with a series sections of known amount of radioactivity (ranging from 0.14 to 63.1 nCi/mg) was counted, and quantitative data analysis with β -Vision Plus yielded a ROI activity number ranging from 0.06 to 21.2 cpm/mm². These data were linearly fitted with a coefficient (R) greater than 0.99 (Fig. 5). This standard curve was used for calibration of tritium autoradiography of tissue sections (i.e., converting cpm/mm into nCi/mg tissue), and the receptor bound radioligand densities were readily calculated utilizing the specific activities of radioligands.

Absolute dopamine D₂ and D₃ receptor densities from in vitro quantitative autoradiography

On the basis of the saturation binding analysis and the in vitro binding data of [³H]WC-10 and [³H]raclopride to cloned human and rat D₂ and D₃ receptors, fractions of D₂ and D₃ receptor occupancies with 4 nM [³H]WC-10 and 10 nM [³H]raclopride binding in monkey and rat caudate-putamen can be readily determined. The values of a_1 , b_1 , a_2 , b_2 , are summarized in Table II. With quantitative autoradiographic analysis, we measured the apparent receptor binding density (B_1) of D₂ plus D₃ receptors using 4 nM [³H]WC-10 in monkey anterior caudate, putamen and rat striatum to be 125, 141, and 38 fmol/mg tissue respectively. The apparent receptor binding density (B_2) of D₂ plus D₃ receptors in monkey caudate, putamen, and rat striatum using 10 nM [³H]raclopride are found to be 318, 345, and 145 fmol/mg tissue, respectively. With these receptor occupancy fraction numbers and the apparent receptor binding densities (B_1 and B_2 values), we obtained the absolute D₂ and D₃ receptors densities from Eqs. 5 and 6. D₂ densities were found to be about 310, 307, and 143 fmol/mg tissue for monkey caudate, putamen, and rat striatum respectively, about 2.1~2.4 fold of D₃ densities. The apparent receptor binding densities of 4 nM [³H]WC-10 and 10 nM [³H]raclopride, absolute D₂ and D₃ receptor densities (D₂ and D₃ values), and their density ratios (D₂/D₃) in rhesus monkey anterior caudate-putamen and rat striatum are summarized in Table III. With the same method, we calculated the absolute D₂ and D₃ receptor densities in monkey post anterior caudate-putamen where globus pallidus was present. We found that D₃ receptor density in post anterior striatum was lower than in anterior striatum and about one-fourth of D₂ receptor density (data not shown).

Comparison of digital beta imager autoradiography and film autoradiography

Autoradiograms of 4 nM [^3H]WC-10 incubated brain slides obtained with the traditional ^3H -Hyperfilm exposure techniques with different exposure period, 1 and 3 months, are shown in Figure 6. Although the image of 3-month exposure had a clear view of striatum and islands of Calleja, the image of 1-month exposure did not show enough signal for visualization. Because the digital Beta Imager can produce high contrast images for quantitative analysis after a 24-h acquisition period (Fig. 4), compared to traditional film autoradiography, the Biospace Beta Imager 2000Z typical standard is a more sensitive, quantitative and higher-throughput tool for receptor autoradiography binding assays with tritium.

DISCUSSION

In a previous report (Xu et al., 2009), we have characterized the D_3 receptor antagonist [^3H]WC-10 and demonstrated it to be a high affinity and moderately selective radioligand for D_3 vs. $\text{D}_{2\text{L}}$ receptors using in vitro radioligand binding and autoradiography studies. WC-10 was radiolabeled with tritium, and the in vitro binding of [^3H]WC-10 to cloned Dopamine $\text{D}_{2\text{L}}$ and D_3 receptors was evaluated. [^3H]WC-10 binds with a 66-fold higher affinity to human HEK D_3 than to HEK $\text{D}_{2\text{L}}$ receptors, with a dissociation constant (K_d) of 1.2 nM. However, [^3H]WC-10 binds to rat Sf9 r D_3 receptors with a K_d of 3.9 nM, a value that is 3-fold lower than binding to human HEK D_3 receptors and a 40-fold value higher than binding to rat Sf9 r $\text{D}_{2\text{L}}$ receptors. In-vitro autoradiography studies show that [^3H]WC-10 labeled the majority of D_3 sites in the caudate and putamen for both adult rats and monkey brains.

In this comparative study, we characterized D_2 selective antagonist [^3H]raclopride binding properties to genetically cloned Dopamine $\text{D}_{2\text{L}}$ and D_3 receptors. [^3H]raclopride binds with a 11-fold higher affinity to human HEK $\text{D}_{2\text{L}}$ ($K_d = 1.6$ nM) than to HEK D_3 ($K_d = 18$ nM) receptors, and [^3H]raclopride binds to rat Sf9 r $\text{D}_{2\text{L}}$ receptors with a K_d of 6.79 nM, a value that is 4-fold lower than its binding to human HEK $\text{D}_{2\text{L}}$ receptors and 2.5-fold higher than its binding to rat Sf9 r D_3 receptors. In vitro quantitative autoradiography studies with the combination of D_3/D_2 ligand, [^3H]WC-10 and the D_2/D_3 ligand, [^3H]raclopride, were also conducted on adult rat and rhesus monkey brain sections. A mathematical model for calculating the absolute densities of Dopamine D_2 and D_3 receptors based on the in vitro binding data obtained from [^3H]WC-10 and [^3H]raclopride was also developed. Although it would be ideal to develop a more selective dopamine D_2 or D_3 receptor ligand for direct measurements of D_2 and D_3 receptors, the assay we presented in this article permits an indirect evaluation of D_2 and D_3 receptor density ratios.

Although the D_3 receptor density was found to be lower (2.1- to 2.4-fold) than D_2 receptor density, and much lower (7- to 8-fold) than the dopamine D_1 receptor or vesicular monoamine transporter 2 (VMAT-2) density in the caudate putamen (unpublished data from our group), the results presented here clearly show extensive binding of [^3H]WC-10 in the caudateputamen, which we believe represents binding to D_3 receptors. These results contradict the binding data obtained with the D_2/D_3 agonist, [^3H]7-OH-DPAT, but they are consistent with immunoprecipitation and immunofluorescence studies with antibodies demonstrating D_1 and D_3 heterodimerization, D_2 and D_3 receptors coexpression in the striatum (Ariano and Sibley, 1994; Boundy et al., 1993; Fauchey et al., 2000; Fiorentini et al., 2008; Marcellino et al., 2008; Schwartz et al., 1998). They are also in agreement with in situ hybridization studies showing extensive distribution of D_3 mRNA in the striatal tissue (Deransart et al., 2001; Joyce et al., 2004; Mihara et al., 2003; Quik et al., 2000). A binding study in D_3 knock-out mice using the D_2/D_3 agonist [^3H]-(+)-PHNO (Rabiner et al., 2007) demonstrated a 50% reduction in ligand binding in the striatum of knock-out vs. wild type mice, suggesting that D_2 and D_3 receptors are expressed with similar densities in the

striatum (Yaroslavsky et al., 2006). Furthermore, in-vitro autoradiography, radioligand binding and positron emission tomography (PET) imaging studies also indicate that D₃ receptors are extensively expressed in the rat, cat, monkey and human striata (Camacho-Ochoa et al., 1995; Joyce et al., 1998; Narendran et al., 2006; Neisewander et al., 2004; Rabiner et al., 2009; Silvers et al., 2006; Sweet et al., 2001; Wade et al., 2001; Wallace and Booze, 1995).

The D₂-like dopamine receptors (D₂, D₃, and D₄ receptors) are believed to be involved in the pathogenesis of several psychiatric and neurological disorders and they may play important roles in schizophrenia, Parkinson's disease, and cocaine addiction. D₂ and D₃ receptors are regulated differently in these central nervous system (CNS) disorders (Abi-Dargham et al., 2000; Martinez et al., 2007; Ryoo et al., 1998; Staley and Mash, 1996). Because of the high amino acid homology and binding domain similarity of D₂ and D₃ receptors, development of highly selective ligands either to D₂ or D₃ receptors for direct evaluation of D₂ or D₃ receptors has been challenging. We have developed a unique procedure for determining absolute D₂ and D₃ receptor densities; this assay requires low radioactivity compared to traditional Scatchard saturation binding with multiple concentrations of single radioligand for measuring receptor density. The method described in this paper took advantage of the less D₂ selective D₂/D₃ ligand [³H]raclopride and the moderately D₃ selective D₃/D₂ ligand [³H]WC-10. This method represents a novel assay to indirectly evaluate D₂ and D₃ receptor densities with single concentrations of each radioligand. Our in vitro characterization and in vivo study showed that WC-10 is a useful probe for investigating the role of dopamine D₃ receptors in behavioral pharmacology. The procedure generated here for measuring changes in absolute densities of dopamine D₃ and D₂ receptors can be used by the neuroscience community in investigating the alteration of D₃ and D₂ receptor density in the variety of CNS disorders.

With agonist binding studies, age and species related differences in D₃ and D₂ receptor density were also reported (Flores et al., 1998; Levant, 1998). It is not clear if these alterations come from a conversion between low and high affinity states, or from receptor upregulation. In the previous study (Xu et al., 2009), we demonstrated that WC-10 can displace agonist [³H]7-OH-DPAT binding in caudate and putamen. With the currently available agonist radiotracers and the novel antagonist binding assays we have described in this study, we would be able to quantitatively investigate the high and low affinity states of dopamine D₂ and D₃ receptors for a better understanding of the alterations of these receptors and their significance in both health and disease (Gonzalez and Sibley, 1995; Levant et al., 1993; Mukherjee et al., 2004; Sibley et al., 1982; Stanwood et al., 2000).

In summary, results from our studies indicate that [³H]WC-10 is an excellent radioligand for dopamine D₃ receptors in vitro. Its weak partial agonist properties make this a unique radiotracer for studying the anatomical distribution and the functional and biological roles of dopamine D₃ receptors in neurological systems. Comparative studies with [³H]WC-10 and [³H]raclopride using the mathematical model described here enable the indirect measurement of the absolute densities of D₂ and D₃ receptors. The mathematical model provided an excellent method for quantifying the D₂ and D₃ receptor density ratio. This will be a valuable tool for studying the change in the ratio of D₂ and D₃ receptors in a variety of neurological disorders where an alteration of dopamine receptors is hypothesized.

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Fig. 1. Chemical structure of [^3H]WC-10. Detailed synthesis scheme was reported previously (Xu et al., 2009).

Fig. 2.

Saturation analysis of the binding of $[^3\text{H}]$ raclopride to cloned D_2 -like dopamine receptors. Direct binding analysis was performed to determine the equilibrium binding affinity of $[^3\text{H}]$ raclopride for human hD_3 and hD_2 (A, B) or rat rD_3 and rD_2 (C, D) receptors. Human receptors were expressed in HEK 293 cells and the rat receptors were expressed in Sf9 cells. Scatchard plots were used to determine the dissociation constants (K_d values). The inset graphs are the Hill plots for determining the Hill coefficient (n_H values). K_d and n_H are presented as mean values $\pm$ SEM for $n = 3$.

Fig. 3. Saturation analysis of the binding of $[^3\text{H}]$ WC-10 to rhesus monkey and rat brain. Direct binding analysis was performed to determine the equilibrium binding affinity of $[^3\text{H}]$ WC-10 binding sites in the rhesus monkey caudate (A), putamen (B), and rat striatum (C). Nonspecific binding was determined from samples which contained $1 \mu\text{M}$ S(-)-Eticlopride. Scatchard plots were used to determine the dissociation constants (K_d values). The inset graphs are the Hill plots for determining the Hill coefficient (n_H values). K_d and n_H are presented as mean values $\pm$ SEM for $n = 3$.

Fig. 4. Quantitative autoradiographic analysis of the binding of [^3H]WC-10 and [^3H]raclopride to rat and rhesus monkey brain. Autoradiograms show neuroanatomical localization of [^3H]WC-10 and [^3H]raclopride binding sites in rhesus monkey (**A, B**) and Sprague-Dawley rat (**C, D**) brain sections. For this study [^3H]WC-10 was used at a concentration of 4 nM (**A, C**) and [^3H]raclopride was used at a concentration of 10 nM (**B, D**). The numbers 1 through 4 in panels (**A**) and (**C**) designate the following CNS anatomical regions: 1, cortex; 2, primate caudate; 3, primate putamen; and 4, rat striatum. (**E**) Autoradiographic image of a [^3H]Microscale, which was counted for 24 h along with the brain sections for the purpose of quantitative analysis.

Fig. 5. Quantitative calibration of in-vitro autoradiography. Calibrated autoradiography typical standard curve obtained by counting a series of tritium standards of a [^3H]Microscale, digitalized image was used to analyze the ROI. This curve was used to convert cpm/mm² to nCi/mg to quantify autoradiography.

Fig. 6. Film autoradiography of the binding of [^3H]WC-10 to rat brain. Autoradiograms obtained with the traditional film exposure techniques with different exposure time, (**A**) 1 month; (**B**) 3 months. The numbers 1 and 2 designate the following CNS anatomical regions: 1, cortex; 2, rat striatum. For this study [^3H]WC-10 was used at a concentration of 4 nM.

TABLE I

Dissociation constants (K_d values) of [^3H]WC-10 and [^3H]raclopride binding to dopamine D_2 and D_3 receptors

Receptors	Radioligand	
	[^3H]WC-10 ^a	[^3H]raclopride
hD _{2L}	76 ± 4	1.6 ± 0.1
hD ₃	1.16 ± 0.1	18 ± 1
D _{2L} /D ₃	65.5	0.09
rD _{2L}	158 ± 8.3	6.79 ± 0.6
rD ₃	3.94±0.19	17.1± 1.7
D _{2L} /D ₃	40.1	0.4

K_d values (nM), presented as mean value ± S.E.M for n = 3, were obtained through saturation binding of [^3H]WC-10 and [^3H]raclopride to a: cloned human D₃ and D_{2L} receptors expressed in HEK cells and b: rat D₃, D_{2L} receptors expressed in Sf9 cells.

^aData were taken from Xu et al. (2009).

TABLE IIFractions of D₂ and D₃ receptors occupancy

	a_1	b_1	a_2	b_2
Monkey	0.05	0.78	0.86	0.36
Rat	0.02	0.5	0.6	0.37

a_1 , b_1 , a_2 , and b_2 represents the receptors occupancy fractions of 4 nM [³H]WC-10 and 10 nM [³H]raclopride binding to dopamine D₂ and D₃ receptors in the monkey and rat striatum. These values were derived from the saturation binding isotherm, Eq. 2, using the K_d values in Table I.

TABLE IIIAbsolute D_2 and D_3 receptor densities density ratios in rhesus monkey caudate, putamen, and rat striatum

	B_1 (fmol/mg) tissue	B_2 (fmol/mg) tissue	D_2 (fmol/mg) tissue	D_3 (fmol/mg) tissue	$D_2:D_3$
Rhesus caudate	125 ± 3	318 ± 17	310 ± 21	141 ± 2	2.20 ± 0.16
Rhesus putamen	141 ± 4	345 ± 23	307 ± 30	130 ± 5	2.36 ± 0.2
Rat striatum	38 ± 2	145 ± 4	143 ± 7	68 ± 3	2.11 ± 0.23

Apparent binding densities (B_1 and B_2) of 4 nM [3 H]WC-10 and 10 nM [3 H]raclopride were directly measured with quantitative autoradiography; absolute D_2 and D_3 receptor densities and the receptor density ratios $D_2:D_3$ were calculated from the Eqs. 5 and 6 using the a_1 , b_1 , a_2 , and b_2 values in the Table II and the B_1 , B_2 values in this Table. Data were presented as mean value ± S.E.M, $n = 8$ for rhesus monkey caudate-putamen and $n = 30$ for rat striatum.