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Characterization of [^{125}I]RTI-121 binding to dopamine transporter *in vitro*[1]

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AIM: To characterize the binding of [^{125}I]3β-(4-iodophenyl) tropane-2β-carboxylic acid isopropyl ester (RTI-121) to the dopamine transporter (DAT) under physiologically relevant conditions. **METHODS:** [^{125}I]RTI-121 was used to label the DAT on fresh rat striatum synaptosomal membranes in artificial cerebrospinal fluid (ACSF) at 37 ℃. **RESULTS:** [^{125}I]RTI-121 binding reached equilibrium within 3 min and remained at its plateau value for at least 9 min. The data from kinetic, saturation, and competition studies supported a one-site model for the binding of [^{125}I]RTI-121 to the DAT. Various DAT blockers (cocaine, GBR12935, and BTCP) and substrates (dopamine and *d*-amphetamine) competitively inhibited the binding of [^{125}I]RTI-121. Compared with NaPhos-KCl-NaCl assay buffer, ACSF containing Ca^{2+} and Mg^{2+} markedly increased the IC_{50} of DAT blockers for inhibiting [^{125}I]RTI-121 binding with less effect on that of substrates. Various D_2 receptor ligands (pergolide, quinpirole, sulpiride, and *l*-stepholidine) had no direct effect on the binding of [^{125}I]RTI-121. **CONCLUSION:** [^{125}I]RTI-121 binding under physiologically relevant conditions fulfills the basic criteria for DAT binding assay.

The neuronal dopamine transporter (DAT) is responsible for synaptic clearance of dopamine[1] and is a major target for abused drugs such as cocaine and *d*-amphetamine, as well as the neurotoxin[2,3]. The interactions between D_2 receptor agents and DAT were observed in binding and translocation *in vitro* studies[4,5]. However, whether such an interaction is direct or indirect remains unclear.

As temperature and ion concentrations exert impacts on the binding of compounds to the DAT[6,7], it is more meaningful to assess the relationship between a compound and DAT under physiologically relevant conditions. However, almost all DAT binding assays have been done under less physiologic conditions, because there are practical problems with the previously developed probes for a DAT binding assay under physiologic conditions: relatively low affinity, rapid dissociation rate, recognition of sites other than those on the DAT, and lower specific activity.

The potent cocaine analogue 3β-(4-iodophenyl)-tropane-2β-carboxylic acid isopropyl ester (RTI-121) was introduced as the most selective probe for labeling DAT in rat brain[8]. The present study was initiated to investigate the characteristics of [^{125}I]RTI-121 binding to the rat striatum synaptosomal membranes under the physiologically relevant temperature, ion concentrations, and pH with attention to the effect of DAT blockers, DAT substrates, and D_2 receptor ligands on the [^{125}I]RTI-121 labeled sites.

MATERIALS AND METHODS

Materials RTI-121 from Research Triangle Institute, USA; [^{125}I]RTI-121 (81.4 GBq·mol^{-1}) from DuPont-New England Nuclear, USA; cocaine hydrochloride from Qinghai Pharmaceutical Co, China; 1-[2-(diphenylmethoxy)ethyl]-4-(3-phenylpropyl) piperazine dihydrochloride (GBR 12935) and *N*-[1-(2-benzo[b]-thiophenyl) cyclohexyl] piperidine (BTCP) from Drs Brian de COSTA, Kenner C RICE, and A E JACOBSON in NIH, USA; mazindol from Sandoz, Switzerland; *d*-amphetamine from SmithKline and French, USA; (−)-quinpirol hydrochloride and S-(−)-sulpiride from Research Biochemical Inc, USA; pergolide mesylate from Lilly Laboratories, USA; *l*-stepholidine from Shanghai Institute of Materia Medica, Chinese Academy of Sciences. All other chemicals from Sigma Chemical Co, USA.

Preparation of synaptosomal membranes Sprague-Dawley rats (♂, $n = 20$, 300 − 350 g) were decapitated. The striata were dissected out and homogenized in 15 vol of ice-cold sucrose 0.32 mol·L^{-1} in a glass homogenizer with a

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Teflon pestle. The homogenate was centrifuged at 1000 × g for 10 min. The supernatant fraction was further centrifuged at 17 000 × g for 20 min. The pellet (P_2) was resuspended in the sucrose. The protein concentration was determined with the Folin phenol reagent method (Lowry *et al*, 1951).

[^{125}I]RTI-121 binding assay Two kinds of assay buffer were used. The first buffer was artificial cerebrospinal fluid (ACSF) and contained NaCl 126, KCl 3, $NaH_2PO_4 \cdot H_2O$ 1, $CaCl_2$ 2, $MgCl_2$ 2, $NaHCO_3$ 20, and glucose 10 mmol·L^{-1}, with pH adjusted by HCl 1 mol·L^{-1} to 7.4 at 37 ℃. The second buffer contained NaH_2PO_4 0.91, Na_2HPO_4 4.54 (pH 7.4 at 37 ℃), NaCl 137, and KCl 3 mmol·L^{-1} (NaPhos-KCl-NaCl). Binding assays were conducted in triplicate in a total volume of 1 or 2 mL. The assay mixture consisting of buffer, [^{125}I]RTI-121 30 pmol·L^{-1}, and membrane protein about 50 mg·L^{-1} was incubated at 37 ℃ for 6 min. The reaction was terminated by rapid filtration through the glass fiber filters presoaked in 0.05 % polyethylenimine on a Multiple Channel Cell Harvester. The filters were washed with three 5-mL portions of ice-cold saline and assayed for radioactivity by a gamma counter (Model FT-613, Beijing Nuclear Instrument Co) with an efficiency of 70 %. The nonspecific binding was defined by cocaine 100 μmol·L^{-1}.

Data analysis IC_{50} values and pseudo-Hill numbers were computed with the equation of the ALLFIT program[9] entered into the Microsoft ORIGIN curve-fitting and plotting software. This nonlinear regression program was run with total and nonspecific binding entered as constants. The data obtained from saturation and kinetic experiments were analyzed with the nonlinear computer fitting program LIGAND for the equilibrium dissociation constant (K_d), the maximal binding capacity (B_{max}), the apparent association rate (k_{obs}) and the dissociation rate (k_{-1}). Because <10 % of the [^{125}I]RTI-121 was bound at equilibrium, the association rate of [^{125}I]RTI-121 (k_{+1}) was calculated with the equation: $(k_{obs} \cdot B_e)/(L_t \cdot R_t)$[10], where B_e is the concentration of [^{125}I]RTI-121 bound at equilibrium, L_t is the total concentration of [^{125}I]RTI-121, and R_t is the total concentration of binding sites as determined in separate saturation experiments. The ratio of $k_{-1}/k_{+1} = K_d$.

All results were expressed as $\bar{x} \pm s$. Statistics consisted of paired and unpaired t tests.

RESULTS

Stability of [^{125}I]RTI-121 binding Preincubation of [^{125}I]RTI-121 with protein-free ACSF for 0, 3, and 8 min before the 6-min incubation with the membranes produced similar specific binding values, but preincubation of [^{125}I]RTI-121 for 20 min resulted in a modest reduction in the binding. Similar results were observed in NaPhos-KCl-NaCl (34.6 and 41.9 nmol/g protein at 0 min, and 27.3 and 31.0 nmol/g protein at 20 min). In contrast, preincubation of brain membranes with radioligand-free ACSF for 0 – 20 min prior to the 6-min incubation with [^{125}I]RTI-121 did not affect the binding (Tab 1).

Tab 1. Effect of preincubation time on [^{125}I] RTI-121 binding in ACSF. $n = 3$. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* time = 0 min (paired t test).

Preincubation with [^{125}I]RTI-121		Preincubation with synaptosomal membranes	
Time, min	Specific binding, pmol/g protein	Time, min	Specific binding, pmol/g protein
0	30.1 ± 2.4	0	31.5 ± 1.9
3	30 ± 5^a	2	30.7 ± 1.9^a
8	30 ± 4^a	4	32 ± 3^a
20	24.4 ± 1.0^c	6	29.5 ± 1.0^a
		10	30.6 ± 1.9^a
		15	29.7 ± 1.9^a
		20	30.6 ± 0.9^a

Kinetic analysis of specific [^{125}I]RTI-121 binding For the association time course, [^{125}I]RTI-121 binding was sampled at various time points between 0.25 and 9 min. The specific binding of [^{125}I]RTI-121 reached plateau levels within 3 min, and stayed essentially constant up to 9 min (Fig 1A). An evident reduction in the binding was observed after 20 min incubation (−23.4 % and −33.3 %). For the dissociation time course, [^{125}I]RTI-121 binding was allowed to establish itself for 6 min and dissociation was initiated by the addition of unlabeled RTI-121 to a final concentration of 1 μmol·L^{-1}. The disappearance of specifically bound radioactivity in time was determined by filtering samples between 0 and 2.5 min. The binding was readily reversed by the addition of excess unlabeled RTI-121 with nearly complete displacement occurring within 3 min (Fig 1A). Both association and dissociation curves were fit to an one-exponential model (Fig 1B and C).

The kinetically derived K_d (k_{-1}/k_{+1}) value was in the same range as the K_d value measured with saturation experiments (Tab 2).

Saturation analysis of specific [^{125}I] RTI-121 binding The analysis was done with both ACSF

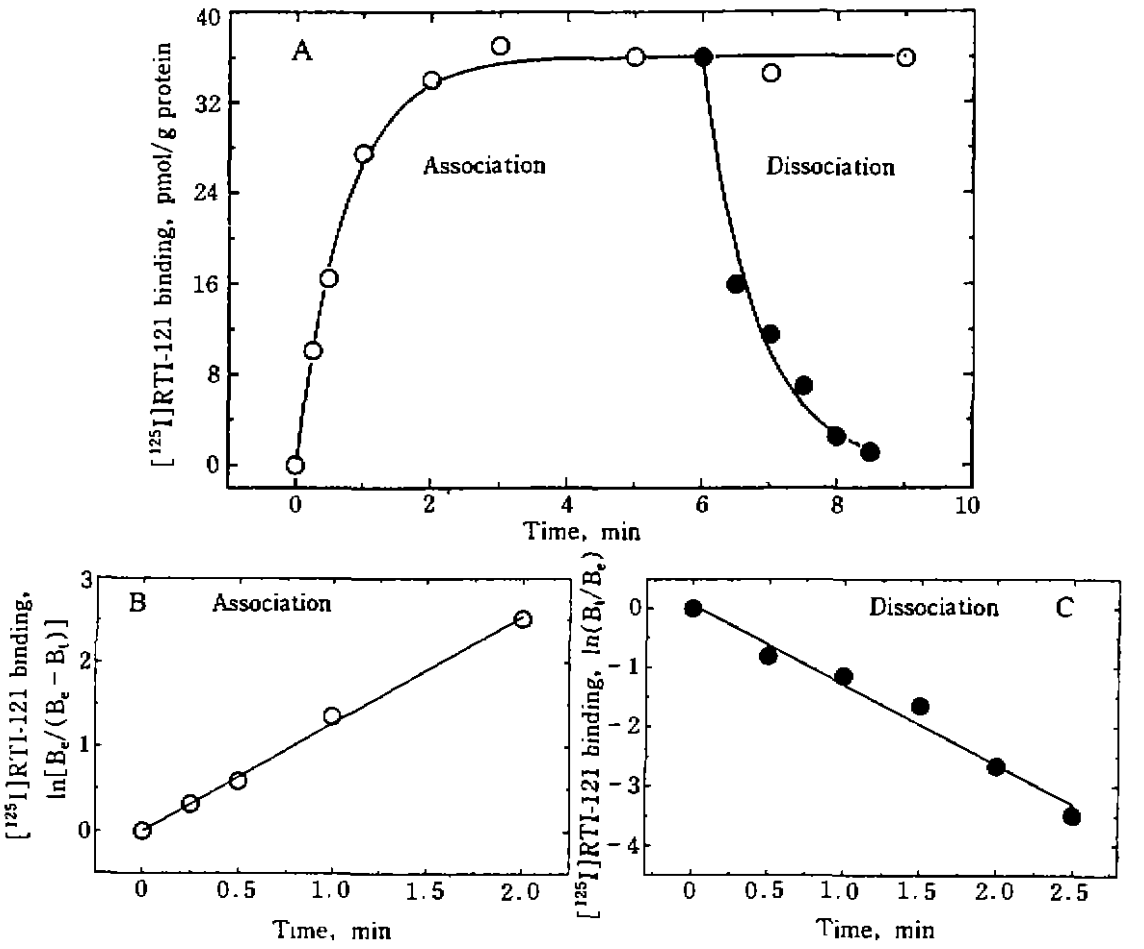

Fig 1. Association and dissociation for [^{125}I]RTI-121 binding in ACSF. A: The line represents the best fit chosen by the LIGAND/KINETIC program to the one-exponential model. B and C: the straight line represents the result of least squares linear regression. Shown are typical experiments, assayed in triplicate, that were performed 3 - 4 times with similar results.

Tab 2. Kinetic constants of specific binding of [^{125}I]RTI-121. n = 3 - 4. $\bar{x} \pm s$. $^{a}P>0.05$, $^{c}P<0.01$ *vs* NaPhos-KCl-NaCl group (unpaired t test).

Constants	Buffer	
	ACSF (n)	NaPhos-KCl-NaCl
k_{+1}, GL·min^{-1}·mol^{-1}	0.16 ± 0.02 (4)	Not detd
k_{-1}, min^{-1}	1.12 ± 0.14 (4)	Not detd
k_{-1}/k_{+1}, nmol·L^{-1}	7.01 ± 0.21 (4)	Not detd
K_i, nmol·L^{-1}	8.3 ± 0.6 (4)^{c}	3.34 ± 0.22 (3)
B_{max}, nmol/g protein	4.29 ± 0.29 (4)^{a}	4.6 ± 0.4 (3)
Hill	1.03 ± 0.03 (4)^{a}	1.05 ± 0.07 (3)

and NaPhos-NaCl-KCl. In saturation studies, "cold method" was used, eg, homotopic displacement of [^{125}I] RTI-121 (30 pmol · L^{-1}) with unlabeled RTI-121 (0 - 100 nmol · L^{-1}). Scatchard analysis of these data showed one binding site (Fig 2).

In agreement with this, the Hill numbers were close to unity (Tab 2).

Incubation of membranes with ACSF reduced the affinity of the DAT for [^{125}I] RTI-121 as compared with NaPhos-KCl-NaCl without changing the number of binding sites (Fig 2 and Tab 2).

Pharmacological analysis of specific [^{125}I]RTI-121 binding In competition studies, 9 concentrations of a DAT blocker or substrate were tested evenly distributed around the IC_{50} values. With both ACSF and NaPhos-KCl-NaCl, various DAT blockers and substrates inhibited [^{125}I]RTI-121 binding in a monophasic fashion with Hill number close to unity. These compounds displaced the bound [^{125}I]RTI-121 completely. The DAT blockers

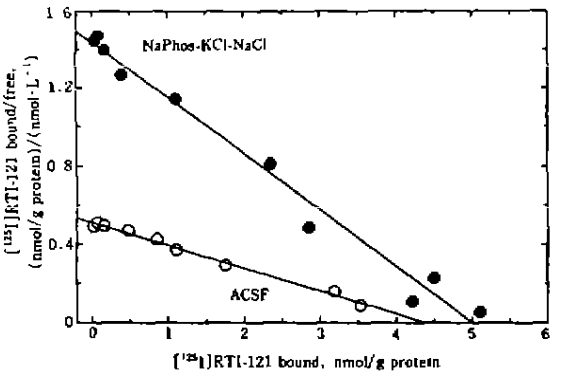

Fig 2. Saturation analysis of [^{125}I] RTI-121 binding in ACSF and NaPhos-KCl-NaCl. The straight line represents the best fit chosen by the LIGAND program. Typical experiments were assayed in triplicate, that were performed 3 – 4 times with similar results.

RTI-121, cocaine, GBR12935, and BTCP showed higher IC_{50} for inhibiting the binding in ACSF than in NaPhos-KCl-NaCl. The IC_{50} of selective DAT blocker GBR12935 was increased by approximately 6 times. The DAT substrate dopamine also showed higher IC_{50} in ACSF than in NaPhos-KCl-NaCl. But this increase was less than that of DAT blockers in terms of fractional increase magnitude. Moreover, there was no significant difference in the inhibitory potency of the substrate *d*-amphetamine between ACSF and NaPhos-KCl-NaCl (Tab 3).

Selective D_2 receptor ligands quinpirole, pergolide, and sulpiride, as well as nonselective DA receptor ligand *l*-stepholidine[11], in a wide range of concentrations, had no effect on the specific [^{125}I]RTI-121 binding in ACSF (Tab 4). With quinpirole 0.1 μmol · L^{-1}, the IC_{50} of DA for inhibiting the binding was 8.6 ± 1.9 μmol · L^{-1} (n = 3), not significantly different from that without quinpirole (see data for dopamine with ACSF in Tab 3).

Tab 4. Effect of D_2 receptor ligands on [^{125}I] RTI-121 binding in ACSF. n = 5, $\bar{x} \pm s$. $^aP > 0.05$ *vs* concentration = 0 (paired *t* test).

Compound	Concentration, μmol·L^{-1}	Specific binding, pmol/g protein
Pergolide	0.00	37 ± 4
	0.01	38.5 ± 1.4^a
	0.10	34 ± 4^a
	1.00	35.9 ± 1.6^a
Quinpirole	0.00	36.2 ± 2.7
	0.01	35.6 ± 1.5^a
	0.10	32.3 ± 2.2^a
	1.00	37.2 ± 1.3^a
Sulpiride	0.00	38.0 ± 1.5
	0.01	40 ± 4^a
	1.00	39 ± 4^a
	10.0	36.8 ± 1.4^a
l-Stepholidine	0	37.6 ± 2.5
	1	38.6 ± 2.1^a
	10	35.8 ± 2.8^a
	100	37.6 ± 1.7^a

DISCUSSION

At odds with the studies carried out at 23 or 25 ℃[8,12], the binding of [^{125}I]RTI-121 decreased appreciably after 20 min incubation at 37 ℃ in our preliminary experiments. This raised a question whether [^{125}I]RTI-121 binding could reach equilibrium under present condition. Therefore, we tested the stability of the radioligand and synaptosomal membranes at 37 ℃. The results indicated that the reduced binding after 20 min

Tab 3. Inhibition of specific [^{125}I]RTI-121 binding by DAT blockers and substrates. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.01$ *vs* NaPhos-KCl-NaCl group (unpaired *t* test).

Compounds	Artifical cerebrospinal fluid			NaPhos-KCl-NaCl buffer			Ratio IC_{50}, $IC_{50,\ ACSF}$/ $IC_{50,\ NaPhos\text{-}KCl\text{-}NaCl}$
	n	IC_{50}, nmol·L^{-1}	Hill	n	IC_{50}, nmol·L^{-1}	Hill	
RTI-121	3	8.20 ± 0.10^b	1.03 ± 0.05	3	3.5 ± 0.4	0.99 ± 0.17	2.3
Cocaine	3	1 134 ± 284^b	0.90 ± 0.09	3	373 ± 36	0.830 ± 0.017	3.0
GBR12935	4	7.6 ± 2.3^b	0.88 ± 0.12	4	1.1 ± 0.4	0.85 ± 0.13	6.9
BTCP	3	36 ± 9^b	1.02 ± 0.10	3	10.4 ± 1.2	0.89 ± 0.08	3.6
Dopamine	4	9 305 ± 1 710^b	0.90 ± 0.10	5	5 660 ± 631	0.90 ± 0.06	1.6
d-Amphetamine	3	4 083 ± 542^a	1.06 ± 0.17	3	3 343 ± 706	0.93 ± 0.08	1.2

incubation was most due to the degradation of [^{125}I]RTI-121 rather than the deterioration of synaptosomal membranes at 37 ℃. According to the k_{-1} for RTI-121 (Tab 2), the dissociation half-life was 0.62 min. After five half-lives (3.1 min), the binding was only 3 % less than the equilibrium value. Since the degradation of [^{125}I]RTI-121 did not occur within 14 min (8 min preincubation plus 6 min incubation with brain membranes), [^{125}I]RTI-121 binding would be at equilibrium with the DAT under present condition.

In previous works with [^{125}I]RTI-121, saturation analysis of the binding revealed a two-component system with a high- and low-affinity site[8,13], or a one-component system[12]. The present study agrees with the latter observation by 3 lines of evidence: one-exponential association and dissociation; linear Scatchard plot of saturation analysis; and monophasic and complete displacement of bound [^{125}I]RTI-121 by various DAT blockers and substrates.

One noticeable discrepancy between the present and previous studies with [^{125}I]RTI-121 is that the K_d was much higher in the present study: approximately 10 times higher in NaPhos-KCl-NaCl, and 20 times higher in ACSF, than that reported by Boja *et al*[8]. Three factors may account for the higher K_d of RTI-121: the unfavorable thermodynamics of radioligand binding[6]; the lack of sucrose known to enhance the affinity of radioligand for the DAT[14]; the presence of inhibitory cations[7]. Because ACSF and NaPhos-KCl-NaCl contained similar concentrations of Na^+ and K^+, and because HCO_3^- has strong stimulatory effect on the binding of cocaine-related radioligands[7], the further increment in the K_d of [^{125}I]RTI-121 observed in ACSF should be attributed to the additional presence of Ca^{2+} and Mg^{2+} in this buffer. In searching for a cocaine antagonist against cocaine abuse, one possible strategy is to look for a compound that potently inhibits binding of a cocaine-related radioligand to the DAT but less affects DA uptake. The present experimental condition for [^{125}I]RTI-121 binding was designed to mimic the condition for determination of DA uptake, hence allowing a more rational evaluation of whether this compound could serve as a cocaine antagonist[15]. In this respect, the presently observed IC_{50} of RTI-121 for inhibiting the binding is close to the previous reported K_i of it for inhibiting [^{3}H]DA uptake[15].

Although both DAT blockers and substrates appeared to inhibit [^{125}I]RTI-121 binding competitively, the affinity of the DAT blockers for [^{125}I]RTI-121 labeled sites declined to a greater degree than that of the substrates when the assay buffer was changed from NaPhos-KCl-NaCl to ACSF containing inhibitory bivalent cations such as Ca^{2+} and Mg^{2+}[7]. It is possible that DAT blockers and substrates recognize nonidentical but overlapping domains on the DAT with the domain of the former more closely associated with the cation sites on the DAT.

In the present study, there was neither direct interaction between D_1/D_2 receptor ligands and [^{125}I]RTI-121 labeled sites nor modulation by D_2 agonist quinpirole of the binding of DA to [^{125}I]RTI-121 labeled sites. These findings, in addition to confirm the high selectivity of [^{125}I]RTI-121 for the DAT under physiological condition, suggest that the previous observed modulation of DA uptake by D_2 agents[5] most likely occurs at the level of DA translocation by occupying D_2 receptors.

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[^{125}I]RTI-121 与多巴胺转运体在体外结合的特征

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关键词 多巴胺；RTI-121 结合；纹状体；突触体；莨菪烷类

目的：研究[^{125}I]RTI-121 与中枢多巴胺转运体(DAT)在体外结合的特征。**方法**：在人工脑脊液(ACSF)中，37 ℃下，用[^{125}I]RTI-121 标记大鼠纹状体突触体膜上 DAT。**结果**：[^{125}I]RTI-121 结合于 3 min 内达到平衡并稳定在峰值至少 9 min。动力学研究、饱和分析和竞争抑制实验均提示[^{125}I]RTI-121 结合到一个位点上。DAT 阻断药可卡因、GBR12935 和 BTCP 以及 DAT 底物多巴胺和苯丙胺均竞争性抑制[^{125}I]RTI-121 结合。与 NaPhos-KCl-NaCl 缓冲液比较，含 Ca^{2+} 和 Mg^{2+} 的 ACSF 显著增加阻断药的 IC_{50}，而对底物的 IC_{50} 影响较小。D_2 受体配基培高利特、quinpirole、舒必利和左旋千金藤立定对[^{125}I]RTI-121 结合无直接作用。**结论**：生理性条件下[^{125}I]RTI-121 结合满足 DAT 结合分析所需的基本标准。