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Structure–activity relationships for substrate recognition by the human dopamine transporter

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Abstract

Information is available on the structure–activity relationships for dopamine as a substrate for uptake by the dopamine transporter. However, dopamine transport is a complex process involving substrate binding, translocation, release as well as transporter reorientation. The present study examines only the substrate recognition step by assessment of the potency of various dopamine-related compounds in inhibiting the binding of the cocaine analog [^{3}H]2β-carbomethoxy-3β-(4-fluorophenyl)tropane ([^{3}H]WIN 35,428) to human dopamine transporters expressed in HEK-293 cells. α-Methylation of the side chain, the presence of the amine, and the 2-carbon-length of the side chain were found to be important for binding affinity, whereas β-hydroxylation of the side chain and methoxylation at the phenyl ring generated weaker compounds. In addition, the presence of both *m*- and *p*-OH at the phenyl ring bestowed an increase in potency but the presence of *p*-OH alone a decrease. N-alkylation (propylation or methylation) had little or an even slightly beneficial effect on affinity, whereas α-carbonylation and α-methanoylation reduced affinity. Amino naphthalene compounds with a fused benzenoid ring system retained some potency consonant with the extended (i.e. β-rotameric) *trans* (=anti) form of the side chain in dopamine when interacting with the transporter. In a second series of experiments, the interaction between dopamine and structural variants was assessed by monitoring the capability of a compound to shift the dopamine inhibition curve to the right as expected for a competitive inhibitor acting at the same site. Appreciable deviation from competitive interaction was observed by removal of the amine from the side chain, by α-carbonylation, and by α-methanoylation. Two blocker-type compounds, semi-rigid variants of cocaine, also displayed significant deviation. A substrate-based compound, inhibiting cocaine analog binding without interfering with dopamine recognition, could be a cocaine antagonist allowing conformational changes to occur during dopamine uptake.

Keywords: Dopamine transport; Dopamine recognition; Dopamine methylation; Dopamine hydroxylation; Dopamine carbonylation; Cocaine antagonist

1. Introduction

The dopamine transporter, serotonin transporter, and norepinephrine transporter, commonly referred to as biogenic amine transporters, clear biogenic amine from extraneuronal fluid in an active uptake process [1]. The uptake cycle is generally thought to consist of separate steps: (1) binding of Na^+, Cl^-, and biogenic amine to external facing form of transporter, (2) conformational change from external to internal facing form or translocation, (3) dissociation of Na^+, Cl^-, and biogenic amine into cytoplasm, and (4) reorientation of transporter to original external facing form [2]. A number of studies have addressed structure–activity relationships for dopamine as a substrate for uptake by the DAT. Early work (1970s) summarized by Horn [3]

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Abbreviations: COCA4A, 8-methyl-(1*R*,*S*)-3-(1-naphthyl)-8-azabicyclo[3.2.1]oct-2-ene; COCA4B, 8-methyl-(1*R*,*S*)-3-(2-naphthyl)-8-azabicyclo[3.2.1]oct-2-ene; DAT, dopamine transporter; DOPAC, 3,4-dihydroxyphenylacetic acid; PHENYL-X, substrate-based cocaine antagonist; WIN 35,428, 2β-carbomethoxy-3β-(4-fluorophenyl)tropane.

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indicated the importance of phenolic hydroxyl groups, α-methylation of the side chain, and extended (β-rotameric, see [4]) *trans* (also referred to as anti) form of the side chain with amine for uptake activity. In contrast, methoxylation, β-hydroxylation and N-methylation reduce uptake activity. More recent work by Meiergerd and Schenk [5] expanded on this information by emphasizing the catechol feature as mediating recognition and the amine side chain as mediating the conformational changes of DAT needed for dopamine translocation. Furthermore, Chen and Justice [6] described the phenethylamine structure as the important structural element accommodated by DAT. In all of the above studies, structural variants of dopamine were tested either as inhibitors of dopamine uptake, or as substrates themselves. Therefore, the uptake activity of a dopamine-related compound reflects the sum of effects of the introduced structural modification on steps 1–3 listed above in the uptake cycle, primarily steps 1 and 2, because step 3 (dissociation) could be extremely rapid [7].

The present study examines only step 1, the recognition step. Compounds with many structural variations related to dopamine were studied for their binding affinity at DAT. Because the dissociation rate of dopamine is too rapid to capture in receptor assays, the dopamine recognition step cannot be measured directly (see [8]). Instead, binding of dopamine and structural variants were assessed indirectly by measuring their potency in inhibiting the binding of [^{3}H]WIN 35,428 which labels DAT with high affinity [9] (see also references in [10]). With this approach one measures the interaction of dopamine with whatever overlap there is between the dopamine and WIN 35,428 binding site or one measures the impact of conformational changes. In addition to the potency of structural variants of dopamine in inhibiting WIN 35,428 binding, the interaction between dopamine and structural variants was assessed by monitoring the capability of a test drug to shift the dopamine inhibition curve to the right as expected for a competitive inhibitor acting at the same site.

2. Materials and methods

2.1. Materials

[^{3}H]WIN 35,428 (85 Ci/mmol) was from NEN Life Sciences Products. Unlabeled WIN 35,428 was from the Research Triangle Institute. Other chemicals were from Sigma–Aldrich, Fisher Scientific, or ICN Biomedicals, Inc. Glass fiber filter mats and Betaplate Scint scintillation cocktail were from Wallac Inc. The cocaine analogs 8-methyl-(1*R*,*S*)-3-(1-naphthyl)-8-azabicyclo[3.2.1]oct-2-ene and 8-methyl-(1*R*,*S*)-3-(2-naphthyl)-8-azabicyclo [3.2.1]oct-2-ene were synthesized at the Department of Chemistry & Pharmacognosy in Chicago (authors M.A. and W.J. D. III) (compounds **4a** and **4b** in [11]).

2.2. [^{3}H]WIN 35,428 binding assay

HEK-293 cells expressing human DAT, graciously provided by the group of Janowsky (see [12]), were grown and used as source for membrane preparations in binding assays as described previously [13,14]. Briefly, culture medium was removed from the cells which were then washed with cold phosphate-buffered saline. Cells were detached from the culture flasks with cold lysis buffer and centrifuged for 20 min at 4° at 31,000 *g*. The supernatant was removed and the pellet was resuspended in ice-cold "Assay buffer" containing 30 mM sodium phosphate (resulting from mixing primary and half-strength secondary sodium phosphate to pH 7.4 at room temperature), 122 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM glucose, 1 mM $CaCl_2$, and 0.1 mM tropolone, with the Brinkmann Polytron (15 s at setting 6). Binding assays were conducted with assay buffer for 15 min at 21° in 96-well plates in a total volume of 0.2 mL with 30–50 μg of the membrane preparation, 3.4 nM [^{3}H]WIN 35,428, and varying concentrations of test compound and/or dopamine, or 100 μM cocaine for defining nonspecific binding. Six concentrations of test compound were used, evenly spaced around its IC_{50} value, with each condition assayed in triplicate. The separation between individual points on the inhibition curves represented an approximately 3-fold difference in drug concentration. Dopamine inhibition curves were constructed with 0.3, 1, 3, 10, 30, and 100 μM of dopamine. Assays were terminated on glass fibre filter mats with the MACH 3-96 Tomtec harvester (Wallac) involving filter washing with phosphate-buffered saline. The filter mats were counted in a Microbeta Plus liquid scintillation counter (Wallac).

As a rule, test compounds were added to binding assays as stocks in water containing 10% (v/v) dimethyl sulfoxide (DMSO) resulting in a final DMSO concentration of 1%. Control assays without test drug (for estimation of total uninhibited binding) also contained 1% DMSO, which by itself had no effect on [^{3}H]WIN 35,428 binding. Dopamine stocks in water were used for the dopamine curves to be tested with and without test compound. Generally, test compounds were weighed out (pipetted if liquid), dissolved in pure DMSO for a final concentration of 100 mM and subsequently diluted out keeping the final DMSO concentration at 10%. Compounds that were very weak in inhibiting [^{3}H]WIN 35,428 binding, i.e. 4-hydroxyphenethyl alcohol, DOPAC, 4-4-hydroxyphenyl acetamide, 4-hydroxyphenyl acetic acid, were dissolved at an initial 500 mM concentration in DMSO. L-Tyrosinol was dissolved at 100 mM in 1 N HCl and then diluted in 10% DMSO; WIN 35,428, cocaine, COCA4A, and COCA4B were dissolved at 10 mM or lower concentration in pure DMSO and subsequently diluted down while keeping DMSO level at 10%. D-Amphetamine was dissolved at 10 mM in water and further diluted in water; in this case, DMSO was also left out from the control assays that had no added drugs.

2.3. Data analysis

IC_{50} values for the inhibition of [^{3}H]WIN 35,428 by dopamine and test compounds were estimated with the ALLFIT equation [15] entered into the ORIGIN nonlinear curve-fitting program. Shifts in dopamine inhibition curves by the presence of a fixed concentration of test compound were calculated as follows. If dopamine, the test compound, and [^{3}H]WIN 35,428 (WIN) compete for the same site, one can write: $IC'_{50} = K_{DA}\ (1 + ([WIN]/K_{WIN}) + ([T]/K_T))$ in which IC'_{50} is the theoretical IC_{50} of dopamine in the presence of fixed concentration of test compound; K_{DA}, K_{WIN}, and K_T are the equilibrium dissociation constants for dopamine, WIN 35,428, and test compound, respectively [16,17]. Under the conditions used here, [WIN] = 3.4 nM, K_{WIN} = 24.6 nM, and $K_{DA\ or\ T}$ was computed from $K_{DA\,or\,T} = IC_{50,\,DA\,or\,T}/(1 + (3.4/24.6))$ [18]. Each 96-well plate contained one curve of dopamine, by itself, and three curves of dopamine with fixed concentration of test compound or curves of test compound alone. Values obtained from the same plate were the basis for pairwise comparison: IC'_{50} was computed from the IC_{50} for dopamine alone with the above equation and compared with the actually observed IC_{50}. In the calculation, a value for K_T was used based on IC_{50} measurements in at least 4–7 separate experiments with test compound. The fixed concentrations of test compound were chosen at IC_{50} values established in preliminary experiments while more results with test compound alone were being obtained along with the dopamine curves. Therefore, the chosen fixed concentrations did not always exactly match the average IC_{50} value. In order to be able to compare compounds in their deviations from the calculated theoretical shifts of dopamine curves based on competitive interaction, the following normalization was used: percent pure competitiveness $= 100 - [(IC'_{50} - \text{observed } IC_{50} \text{ with T})/(IC'_{50} - \text{observed } IC_{50} \text{ without T})] \times 100$.

Theoretical and observed IC_{50} values for dopamine were compared with the paired Student's *t*-test. Because dopamine curves with three test drugs were compared with one curve of dopamine alone per plate, the Bonferroni correction for multiple comparisons (3) was applied to *P* such that the accepted level of significance *P* was 0.05: 3 = 0.016.

3. Results

3.1. Affinity for DAT

Dopamine and many structural variants (Fig. 1) displayed a wide range of affinities for DAT (Table 1, second column). For the majority of the compounds including dopamine the Hill numbers (Table 1, third column) were close to unity, consonant with a competitive mechanism of inhibition. Exceptions with Hill numbers ≥1.4 were 4-ethylcatechol and DOPAC. For all dopamine inhibition curves in the presence of a fixed concentration of test compound, Hill numbers were close to unity with an average SEM of 0.05.

Compared with dopamine (4.30 μM), removal of the catechol feature on the phenyl ring (β-phenethylamine, 18.9 μM) reduced some affinity, counteracted by improved affinity from α-methylation of the side chain (D-amphetamine, 6.33 μM). Removal of the *m*-hydroxyl from the phenyl ring (*p*-tyramine, 34.0 μM) reduced the affinity compared with dopamine, which has both *m*- and *p*-OH, but also compared with β-phenethylamine which has none. The presence of both *m*- and *p*-OH also improved affinity of epinephrine (27.2 μM) over that of synephrin (160 μM) which has only *p*-OH. *m*-Methoxylation was deleterious to affinity (3-methoxytyramine, 272 μM).

Shortening of the side chain (3,4-dihydroxy-benzylamine, 1.00 mM) severely curtailed affinity. The amine on the side chain appeared crucial: all compounds lacking the amine were weak at DAT (4-ethylcatechol, 358 μM; 4-hydroxyphenethyl alcohol, 1.57 mM; DOPAC, 4.02 mM; 4-hydroxybenzyl cyanide, 976 μM; 4-ethoxyphenol, 737 μM). α-Carbonylation (4-hydroxyphenyl acetamide, 5.16 mM; 4-hydroxyphenyl acetic acid, 4.49 mM) also severely interfered with affinity, whereas α-methanoylation had a much less adverse effect (compare L-tyrosinol, 95.9 μM, with *p*-tyramine, 34.0 μM). β-Hydroxylation reduced affinity (compare norepinephrine, 50.0 μM, with dopamine, 4.30 μM). Alkylation of the amine was neutral to affinity or even slightly beneficial (*N*,*N*-di-*n*-propyldopamine, 1.18 μM; compare epinephrine, 27.1 μM, with norepinephrine, 50.0 μM). Lengthening of the side chain, as in serotonin (270 μM) or 2-aminonaphthalene (38.8 μM) reduced affinity compared with dopamine although other structural features were additionally different: the indole in serotonin, conformationally restricting the side chain, and the fused benzenoid ring system in 2-aminonaphthalene which was also present in 1-naphthalene methylamine (52.5 μM). Still, compared with the basic structure of β-phenthylamine (18.9 μM), the activity of the amine containing naphthalenes tested here was rather impressive.

3.2. Ability to competitively shift dopamine curve

Compounds differed in their ability to shift the dopamine inhibition curve to the right as expected from competitive inhibitors acting at the same site acted upon by dopamine and the radioligand [^{3}H]WIN 35,428 (Table 1, last two columns). Examples of competitive inhibitors were dopamine itself and *p*-tyramine, which, when applied together with dopamine (Fig. 2B and D) at the IC_{50} level determined from inhibition curves for dopamine alone (Fig. 2A) or *p*-tyramine alone (Fig. 2C), shifted the dopamine curves to the right approximately 2-fold as expected. In contrast, examples of compounds hardly capable of

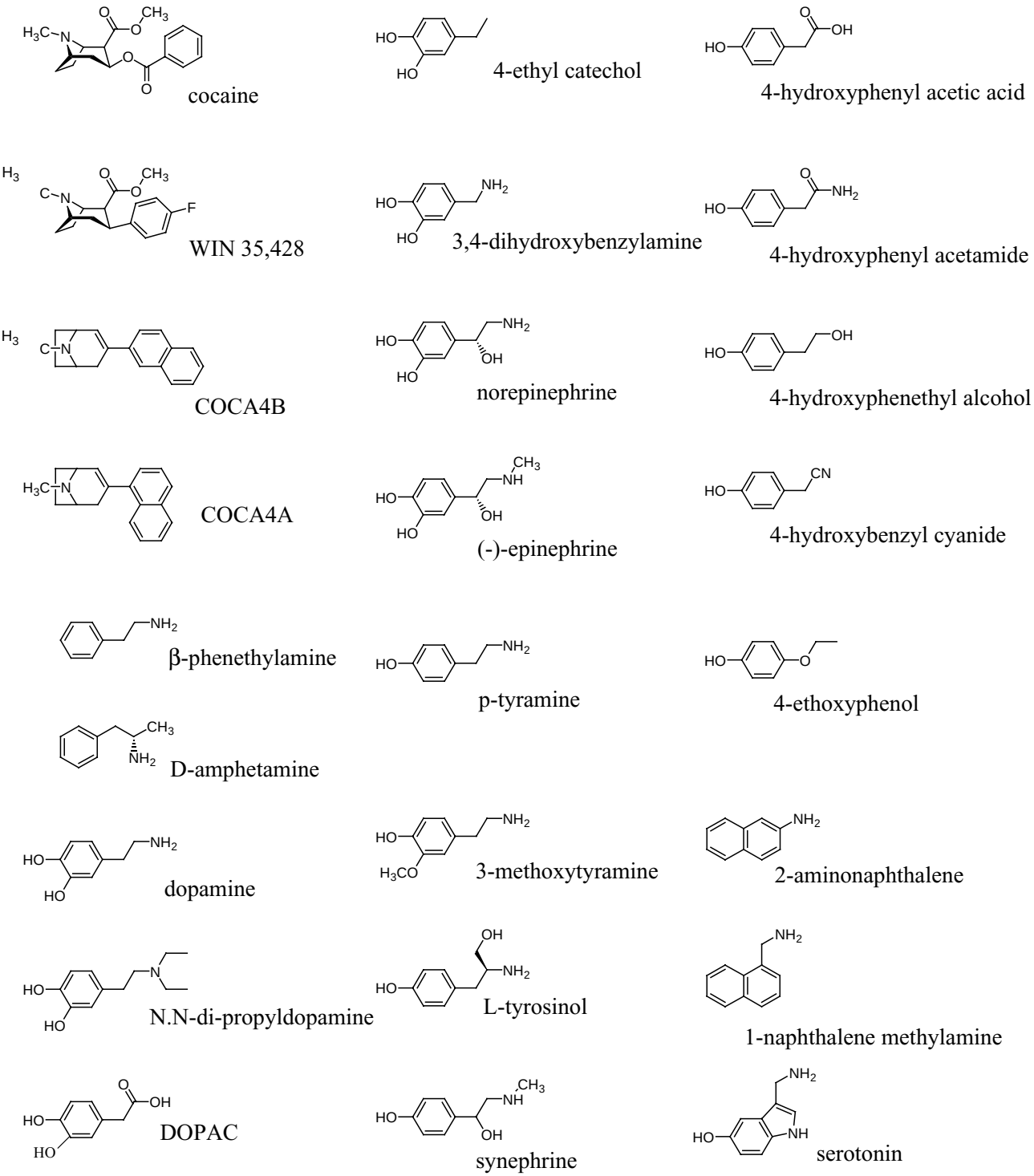

Fig. 1. Structures of compounds studied.

shifting the dopamine curve were DOPAC (Fig. 2E and F) and 1-naphthalene methylamine (Fig. 2G and H). Comparison of deviations from competitive interaction was done based on normalized data (Fig. 3), facilitating comparison of all compounds. Percent pure competitiveness was defined as $100 - [(IC'_{50} - \text{observed } IC_{50} \text{ with T})/(IC'_{50} - \text{observed } IC_{50} \text{ without T})] \times 100$. Thus, in this analysis, 100% pure competitiveness denotes a full shift in the dopamine curve to the right as expected in the competitive case (observed IC_{50} with T equals IC'_{50}; shift is 2-fold if $[T] = IC_{50,T}$); 0% denotes no shift at all (observed IC_{50} with T equals observed IC_{50} without T); and values between 0 and 100% indicate shifts to the right less than expected in the competitive case. Values greater than 100% signify shifts to the right greater than calculated based on the competitive case, whereas negative values denote shifts to the left. As

Table 1
Inhibition of [^{3}H]WIN 35,428 binding by test compound, and ability of test drug to shift dopamine inhibition curve

Test drug (N)[a]	IC_{50}[b] test drug	Hill number	Added [test drug] with DA	IC_{50} DA (μM)	IC_{50} DA (μM) with test drug	
					Theoretical	Observed
Dopamine (6) (μM)	4.30 ± 0.27	0.96 ± 0.08	5.0	4.30 ± 0.27	8.91 ± 0.27	7.38 ± 0.41
WIN 35,428 (4) (nM)	24.3 ± 3.7	1.05 ± 0.08	20	4.68 ± 0.66	8.55 ± 1.20	9.88 ± 2.14
Compound COCA4B (5) (nM)	51.2 ± 8.5	1.09 ± 0.08	51	5.25 ± 0.46	10.5 ± 0.9	6.98 ± 1.03*
Compound COCA4A (5) (nM)	605 ± 66	0.94 ± 0.18	600	5.25 ± 0.46	10.5 ± 0.9	7.21 ± 0.86*
Cocaine (4) (nM)	171 ± 14	1.02 ± 0.05	160	4.68 ± 0.66	9.08 ± 1.28	9.44 ± 1.94
N,*N*-Di-*n*-propyldopamine (9) (μM)	1.18 ± 0.17	1.10 ± 0.04	0.60	5.09 ± 0.64	7.66 ± 0.97	8.72 ± 1.02
D-Amphetamine (4) (μM)	6.33 ± 0.63	0.87 ± 0.08	6.0	5.10 ± 0.83	9.93 ± 1.62	8.61 ± 1.47*
β-Phenethylamine (4) (μM)	18.9 ± 1.7	0.88 ± 0.10	20	4.66 ± 0.63	10.6 ± 1.5	8.09 ± 0.96
Epinephrine (10) (μM)	27.1 ± 3.6	0.94 ± 0.03	28	6.62 ± 0.74	12.8 ± 1.7	10.6 ± 1.2
p-Tyramine (9) (μM)	34.0 ± 5.4	0.96 ± 0.10	50	5.51 ± 1.19	13.6 ± 2.9	14.6 ± 3.6
2-Amino naphthalene (5) (μM)	38.8 ± 9.5	0.83 ± 0.15	39	7.44 ± 1.29	14.9 ± 2.6	8.91 ± 1.68*
Norepinephrine (5) (μM)	50.0 ± 4.8	1.08 ± 0.07	50	6.64 + 1.49	13.3 + 3.0	10.8 + 2.8
1-Naphthalene methylamine (5) (μM)	52.5 ± 13.4	0.89 ± 0.20	52	7.06 ± 1.36	14.1 ± 2.7	8.01 ± 2.12*
L-Tyrosinol (5) (μM)	95.9 ± 10.2	1.02 ± 0.05	96	5.97 ± 0.66	11.9 ± 1.3	7.16 ± 0.87*
Synephrin (6) (μM)	160 ± 25	1.00 ± 0.07	200	5.70 ± 0.73	13.6 ± 1.5	13.7 ± 1.9
Serotonin (6) (μM)	270 ± 26	1.00 ± 0.04	270	7.57 ± 1.10	15.2 ± 2.2	10.6 ± 1.6*
3-Methoxytyramine (7) (μM)	272 ± 18	1.04 ± 0.01	300	4.69 ± 0.70	9.86 ± 1.48	9.13 ± 1.17
4-Ethylcatechol (10) (μM)	358 ± 66	1.43 ± 0.06	600	6.22 ± 0.95	16.6 ± 2.5	8.79 ± 0.90*
4-Ethoxy phenol (5) (μM)	737 ± 143	0.88 ± 0.09	740	5.25 ± 0.46	10.5 ± 0.9	2.75 ± 0.28*
4-Hydroxybenzyl cyanide (6) (μM)	976 ± 168	0.93 ± 0.12	960	7.00 ± 1.26	13.9 ± 2.5	8.67 ± 1.31*
3,4-Dihydroxy-benzylamine (9) (mM)	1.00 ± 0.11	1.01 ± 0.12	1.0	6.07 ± 1.08	11.7 ± 2.0	9.49 ± 1.87
4-Hydroxyphenethyl alcohol (6) (mM)	1.57 ± 0.26	0.83 ± 0.04	2.0	6.37 ± 0.66	14.5 ± 1.5	12.1 ± 1.5*
DOPAC (8) (mM)	4.02 ± 0.18	1.71 ± 0.15	3.0	7.23 ± 1.11	11.4 ± 1.7	7.45 ± 1.00*
4-Hydroxyphenyl acetic acid (6) (mM)	4.49 ± 0.65	1.24 ± 0.16	5.0	5.81 ± 0.45	12.3 ± 1.0	7.39 ± 0.57*
4-Hydroxyphenyl acetamide (7) (mM)	5.16 ± 1.12	0.84 ± 0.12	5.0	6.22 ± 0.43	12.2 ± 0.8	9.18 ± 0.86*

Concentration of [^{3}H]WIN 35,428 (3.4 nM) was well below K_d (24.6 nM). Test drug or dopamine were tested by themselves (2nd and 5th column), or test drug was added at fixed concentration (4th column) along with varying concentrations of dopamine (6th and 7th columns). The N value listed in 1st column also applies to 5–7th column.

[a]N: pairs of theoretical and observed IC_{50} values assessed.

[b]Mean ± SEM for 4–7 experiments.

*$P < 0.016$ compared with theoretical IC_{50} (paired Student's *t*-test with Bonferroni correction).

shown in Fig. 3, a number of compounds displayed percent pure competitiveness ranging from 60 to 140% (dotted lines in Fig. 3), with only D-amphetamine and 4-hydroxyphenethyl alcohol close to the 60% mark exhibiting observed IC_{50} values statistically significantly different from the theoretical IC'_{50} values, while all other compounds close to the 60% mark did not show statistical significance. Compounds showing less than 60% pure competitiveness were COCA4B, COCA4A, 2-amino naphthalene, 1-naphthalene methylamine, L-tyrosinol, serotonin, 4-ethylcatechol, 4-ethoxyphenol, 4-hydroxybenzyl cyanide, DOPAC, 4-hydroxyphenyl acetic acid, and 4-hydroxyphenyl acetamide.

4. Discussion

4.1. Affinity for DAT: comparison of binding and uptake experiments

The current affinity pattern coincides in many respects with the general structure–activity relationships advanced for dopamine from uptake experiments [3,5,6]. Thus, α-methylation of the side chain, the presence of the amine, and the 2-carbon-length of the side chain are important for both binding affinity and uptake activity; β-hydroxylation of the side chain and methoxylation at the phenyl ring generate weaker compounds in both binding and uptake assays. Equivocal results with uptake measurements have been reported regarding the role of phenolic hydroxyl groups: Horn [3], Meiergerd and Schenk [5] point out the importance of the presence of such groups for activity, in particular the *m*-OH in the latter study, whereas Chen and Justice [6] found similar uptake activities regardless of the presence of *m*-OH, *p*-OH, or di-*m*,*p*-OH. The present affinity measurements show compounds with both hydroxyls to be more potent than compounds with *p*-OH alone or compounds that lack both *p*- and *m*-OH; introduction of *p*-OH alone decreases affinity. Another difference between binding and uptake results is apparent when considering the effect of N-alkylation. N-Alkylation (propylation or methylation) in the present binding experiments has little effect on binding potency or actually enhances it, whereas N-alkylation (propylation or methylation) in uptake experiments decreases activity [3,5]. Other features, not previously examined in uptake experiments, were assessed in the present binding experiments: presence of α-carbonyl (deleterious to affinity), introduction of α-methanoyl (reducing affinity), and addition of second phenyl ring,

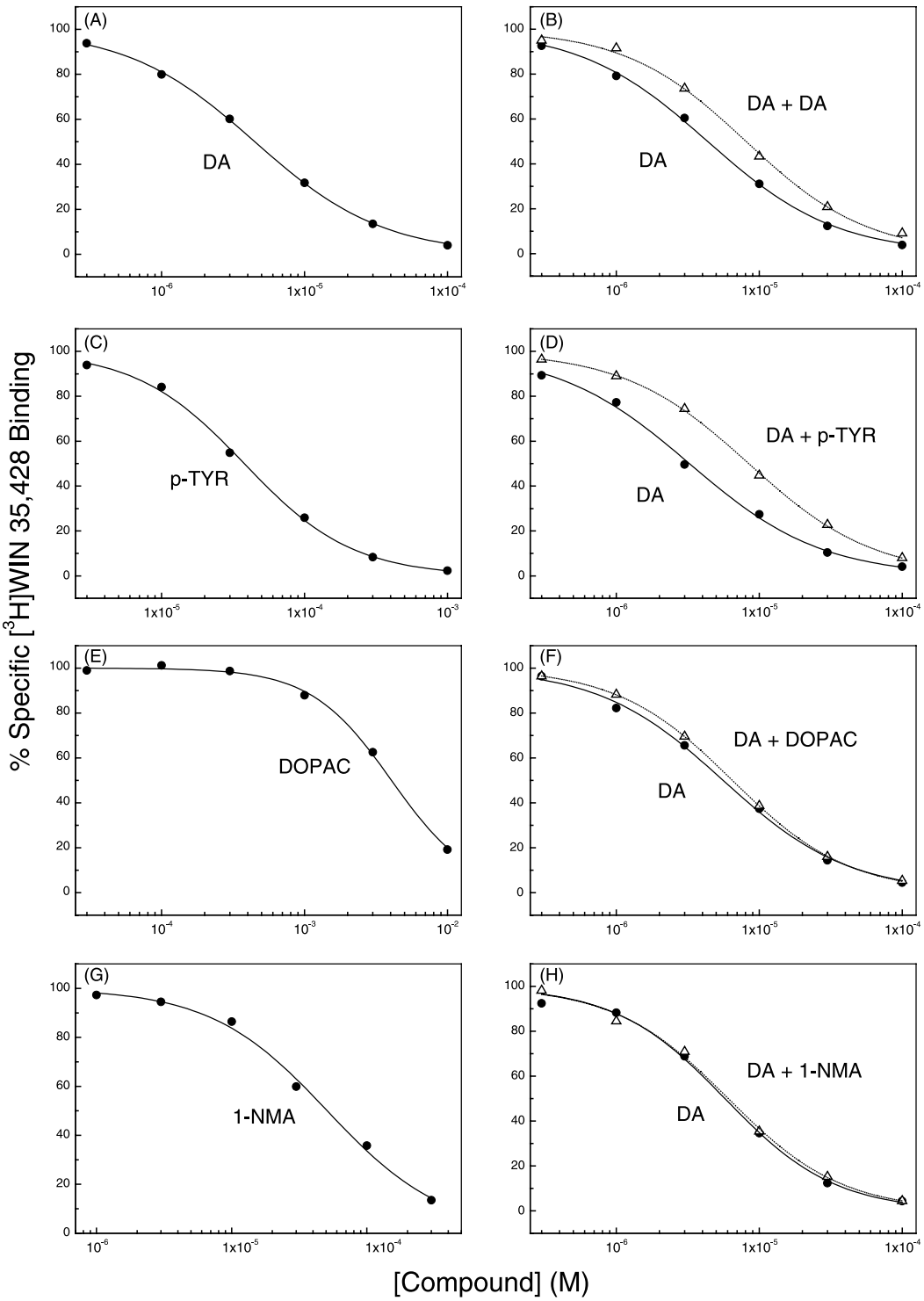

Fig. 2. Inhibition of [^{3}H]WIN 35,428 binding by test compound alone (left panels) or by dopamine (DA) in the presence of a fixed concentration of test compound (DA; *p*-TYR = *p*-tyramine; DOPAC; 1-NMA = 1-naphthalene methylamine) (right panels). The concentration of [^{3}H]WIN 35,428 was 3.4 nM, and the fixed concentrations of test compounds were as listed in Table 1. On the right hand side, the solid curves are for DA alone and the dotted curves for DA plus fixed concentration of test compound.

merged to first ring in amino naphthalenes (retaining some binding potency). The latter result with the amino naphthalenes is interesting and consonant with the finding that the side chain in dopamine is in the extended (i.e. β-rotameric) *trans* (=anti) form [3–5].

Clearly, dopamine uptake is a complex process involving substrate recognition, substrate translocation, and transporter reorientation. Generally, investigators interpret an uptake K_m for a substrate as reflecting the affinity of the substrate for the transporter, and an uptake V_{max} for a

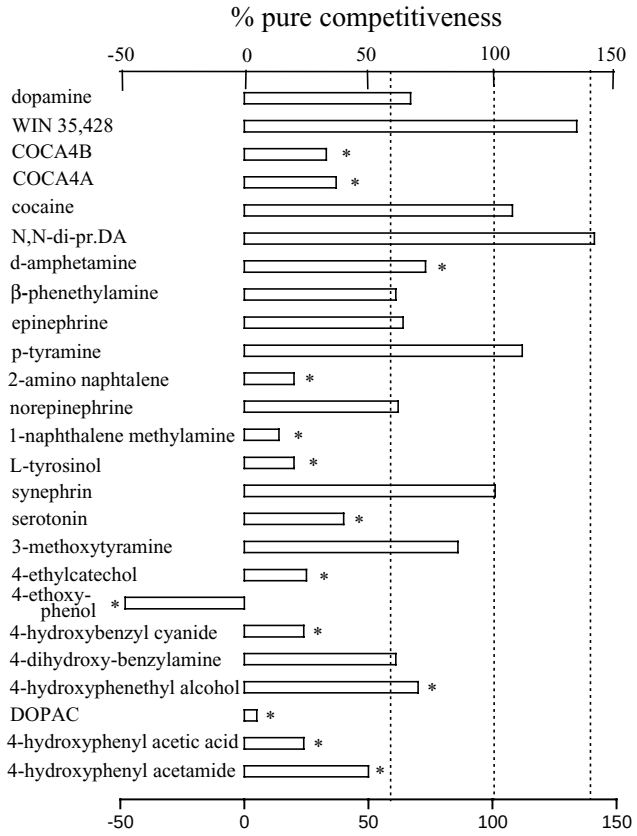

Fig. 3. Percent pure competitive interaction with dopamine. Shifts in dopamine inhibition curves by test compound (fixed at its approximate IC_{50} value) were compared with the theoretical shifts predicted based on the test compound and dopamine interacting at the same site on DAT that binds [^{3}H]WIN 35,428. Percent pure competitiveness was computed as detailed in Section 2. 100% pure competitiveness (dotted, middle straight line) denotes a full shift in the dopamine curve to the right as expected in the competitive case; 0% denotes no shift at all; and values between 0 and 100% indicate shifts to the right less than expected in the competitive case. Values greater than 100% signify shifts to the right greater than calculated based on the competitive case, whereas negative values denote shifts to the left. Most compounds in the 60 to +140% range (dotted straight lines) showed no statistical significance in deviating from competitiveness. (*) $P < 0.016$ (paired, Bonferroni corrected, Student's *t*-test as in Table 1). *N*,*N*-di-pr.DA denotes *N*,*N*-di-*n*-propyldopamine.

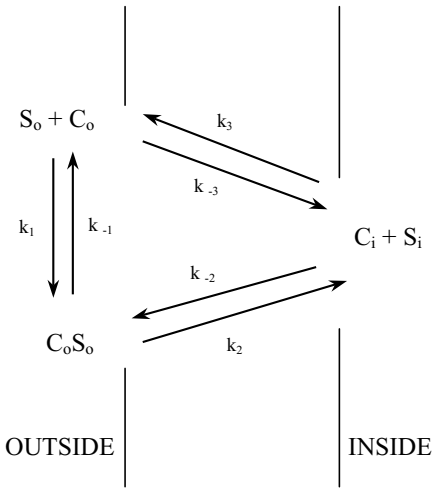

Fig. 4. Translocation model as described by Schömig *et al.* [7]. Externally facing carrier C_o binds substrate S_o resulting in complex C_oS_o (rate constants k_1 and k_{-1}). The complex is translocated towards interior side (rate constant k_2), where substrate (S_i) is released into the cytosol. The empty carrier (C_i) reorients to the outside (C_o) (rate constant k_3). The rate constants k_{-2} and k_{-3} complete the full equilibria in the model.

substrate resulting from translocation. Thus, Meiergerd and Schenk [5] concluded that 4-ethylcatechol (K_m comparable to that of dopamine), is recognized normally, but translocated more slowly with a V_{max} considerably less than that of dopamine; in contrast, 3-methoxytyramine (K_m seven times higher than that of dopamine but normal V_{max}) is considered to be recognized poorly but translocated normally. IC_{50} values of substrates can be measured with substrates added in low concentrations as co-substrates with dopamine in uptake assays, and these IC_{50} values reflect their K_m values [19]. Horn [3] discusses these values as representing ''the affinity for the neuronal uptake site''. Nevertheless, transport models that have been developed taking into account the various steps in the uptake cycle, point to a number of kinetic constants that together describe K_m; only one of those is the affinity in the recognition step [19,20]. Furthermore, a number of kinetic constants describe V_{max}; only one of those is the rate constant for substrate translocation [19,20]. A simplified model as described by Schömig *et al.* [7] takes into account substrate recognition (equilibrium dissociation constant K_S), translocation of loaded carrier (rate constant k_2), and transporter reorientation (rate constant k_3), while ignoring release of substrate internally as an extremely rapid step (Fig. 4). From this model it can be derived [21] that both the K_m and V_{max} expression includes a term (k_3/[$k_3 + k_2$]), i.e. kinetic rate constants, not only K_S, impact K_m; also, the reorientation rate constant, not only the translocation rate constant, impacts V_{max}. The full expressions are: $K_m = K_S\ (1 + k_2/k_{-1})(k_3/[k_3 + k_2])$ and $V_{max} = C_{TOT}.\ k_2(k_3/[k_3 + k_2])$ in which k_{-1} = dissociation rate for external substrate binding and C_{TOT} = total concentration of carrier [21]. As V_{max} values can differ between DAT substrates [5,19], different substrates can be expected to have varying values of k_2 (for loaded carrier), thereby potentially affecting K_m. This most likely underlies, at least in part, the differences noted between binding and uptake results. For example, 4-ethylcatechol is recognized by DAT with appreciably lower affinity than dopamine (Table 1) even though the K_m of 4-ethylcatechol is comparable to that of dopamine [5]. This can be understood taking into account the lower V_{max} for 4-ethylcatechol compared with dopamine. The lower V_{max} could be the result of a decrease in k_2 which in turn could decrease K_m as a fraction of K_S: thus, even with a higher K_S for 4-ethylcatechol compared with dopamine, its K_m could be comparable. Clearly, K_m reflects more than the impact of K_S.

In comparing uptake and binding measurements, it is to be noted that uptake is assessed in intact cells (or nerve endings in tissue) and binding in broken membrane

preparations. Although the Na^+ dependency of dopamine binding has been shown to differ between intact cells and broken membranes, the underlying mechanism appears to be the access of dopamine to its binding site on the DAT protein, with dopamine's affinity being equal in the two preparations when Na^+ is present at extracellular level [22]. The present affinities measured in membranes therefore likely provide information regarding recognition in intact cell preparations. However, more information is needed to ensure that the binding potencies of substrates other than dopamine are not affected by the preparation used as source for the DAT.

4.2. Deviation from competitive interaction with DA: cocaine antagonism?

In the following, a competitive mechanism of inhibition of [^{3}H]WIN 35,428 binding by test compounds, including dopamine, when present by themselves, is inferred from the observation that the Hill numbers for their inhibition curves are close to unity, with the exception of the case for 4-ethylcatechol and DOPAC with Hill numbers $\geq$1.4. In addition, in the following discussion, caution is exercised in not overinterpreting the shifts in the dopamine curves with test compound compared with the dopamine alone curves. Because the bulk of the compounds falling in the range of 60–140% pure competitiveness did not show statistical significance (Fig. 3), only those compounds that displayed less than 60% pure competitiveness were considered deviant. Finally, because the compounds deviating from competitiveness cover a wide range of affinities, from 50 up to 5 mM, we speculate that the deviation is not caused by some nonspecific mechanism associated with low-affinity binding.

One way to consider substrate recognition by such deviant compounds could be inspired by the classical lock-and-key model. With this model in mind, one would need to view deviation from competitive interaction between a test compound and dopamine as evidence for the two compounds acting on distal sites. In contrast, sharing a binding site or acting at overlapping sites would necessarily lead to competitive interaction. In this view, one could consider how the *addition of an extra group* to a dopamine-like structure would affect its recognition compared with dopamine. Such a group could be bulky, sterically hindering the docking of the compound into its normal site, or have an electrical charge being repulsed by an opposite charge at DAT, resulting in the compound binding at a distal site with lower affinity. This could be the case with α-carbonylation (4-hydroxyphenyl acetamide, 4-hydroxyphenyl acetic acid) or α-methanoylation (L-tyrosinol) which drastically reduce affinity. If the additional group is not in the way sterically or is inert, or even if the group forms a new point of interaction while docking in its site at DAT, the structural analog would be expected to be a competitive inhibitor of dopamine's effect on WIN 35,428 binding just like dopamine itself. This could be the case for α-methylation (D-amphetamine) or N-alkylation (*N*,*N*-di-*n*-propyldopamine, synephrin).

Removal of a group from the dopamine structure could be thought to have a profound effect in the competition experiment if the group plays a role in interacting with DAT. For example, because 4-ethylcatechol lacks the amine, it may not be able to bind to its normal site with the catechol by itself perhaps being unable to sustain docking in the site; the result could then be the occurrence of binding to a distal site with lower affinity. On the other hand, if the group is not important for interaction with the normal site, its removal should not alter the behavior of the compound as a competitive inhibitor of dopamine action. In this way of reasoning, the hydroxyls at the phenyl ring appear to contribute to affinity for docking in the normal site, but their removal (*p*-tyramine, β-phenethylamine) still allows the phenyl ring with amine carrying side chain to bind to the site, albeit with lower affinity, and still allows competitive interaction with dopamine.

The same lock-and-key model underlies the concept of a dopamine-sparing cocaine antagonist [23–26]: such a compound would inhibit cocaine (analog) binding but not interfere with dopamine recognition (Fig. 5). A cocaine antagonist based on inhibitor structures such as cocaine or other blockers, with appreciable affinity for DAT, has been elusive perhaps because such a blocker-based antagonist holds the transporter in a conformation not allowing transport. Possibly, a substrate-based cocaine antagonist (PHENYL-X, Fig. 5) could prevent the action of cocaine while allowing conformational changes to occur during dopamine uptake. PHENYL-X could be a substrate-derived inhibitor lacking the amine (DOPAC, 4-ethylcatechol, 4-ethoxyphenol) or catechol (amino naphthalenes, 4-hydroxyphenyl acetamide). Among those, the naphthalenes have the better affinities. Ideally, PHENYL-X would not be taken up by

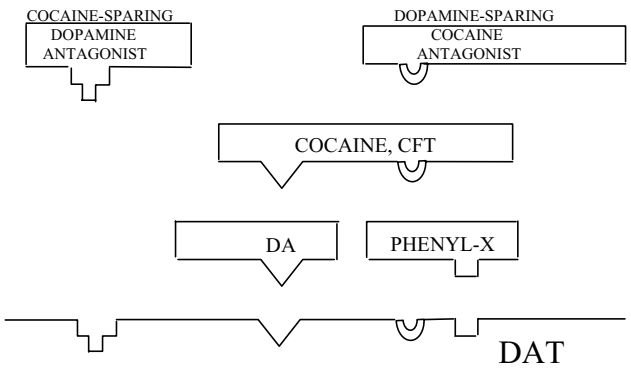

Fig. 5. Cartoon of binding domains on DAT for substrates, blockers, and antagonists according to lock-and-key model. The dopamine-sparing cocaine antagonist is indicated as sharing a point of contact with DAT with cocaine but could also be visualized as a case of overlap. As a corollary only, a cocaine-sparing dopamine antagonist is depicted, overlapping with DA. PHENYL-X, just like a dopamine-sparing cocaine antagonist, could interfere with the binding of cocaine but not DA while allowing conformational changes occurring during DA transport (see text).

DAT, and indeed both DOPAC and 4-ethylcatechol are known to be not transported well by DAT [5]. Although DOPAC and 4-ethylcatechol displayed Hill numbers appreciably different from unity (Table 1), their deviation from competitiveness may be unrelated to this as many other deviant compounds had normal Hill numbers close to unity.

One problem with the lock-and-key model discussed above is that, theoretically, the predictions for distally binding compounds go farther than a mere deviation from competitive interaction. When the test compound and dopamine act at distal sites, both of which are mutually exclusive with WIN 35,428, the presence of a fixed concentration of test compound at its IC_{50} level can be calculated to actually enhance the potency of dopamine in inhibiting WIN 35,428 binding, i.e. shifting the dopamine curve to the left, rather than merely shifting the dopamine curve to the right less than 2-fold. This has been demonstrated in the classical paper of Fairhurst *et al.* [27] as well as Huang and Ehrenstein [28] for sodium and calcium channel blockers. The same papers also detail the ''intermediate'' situation which, as applied to our case, has the test compound compete for the dopamine site in addition to acting at a distal site (with both sites being mutually exclusive with WIN 35,428). Theoretically, this situation can result in no shift at all in the dopamine curve in the presence of test compound, and a number of compounds in the present study actually appeared to behave in this manner.

Another issue that needs to be addressed in considering the lock-and-key model, is that, in the few cases of proposed cocaine antagonist activity, 7α-methoxycocaine [16]; various local anesthetics, antipsychotics, other drugs [29]; and a methylphenyl-substituted piperidyl-methylphenyl-ketone [26], did interact weakly with dopamine action as measured in dopamine uptake assays, while interfering more strongly with cocaine (analog) binding as evidenced in experiments assessing inhibition by cocaine of dopamine uptake (strong shifts of cocaine curves to the right) or inhibition of cocaine analog binding (high potency). In order to explain the weak interaction between dopamine and the cocaine antagonist, one would have to invoke additional mechanisms, such as ''partial overlap'' or ''partial sharing'' as proposed to explain similar observations regarding the interaction between N-ethylmaleimide and DAT compounds [30]. Even more likely, additional conformational changes may play crucial roles in the interaction between compounds and DAT. Thus, it is becoming increasingly likely that the binding of compounds such as dopamine and cocaine to DAT induce conformational changes that affect the accessibility of various domains of the DAT protein [31–33].

4.3. Comparison of profile in affinity and competition tests: measuring different aspects

In some cases there is agreement between tests for affinity and assessments of competition with dopamine regarding the question of which groups play an important role for interaction with DAT. For example, the amine on the side chain appears crucial in both tests, with weak affinity and lack of competitiveness displayed by 4-ethylcatechol and 4-hydroxybenzyl cyanide which do not carry the amine. α-Carbonylation (4-hydroxyphenyl acetamide) or α-methanoylation (L-tyrosinol) decreases affinity and leads to deviation from competition, possibly because the extra group obstructs docking of the ligand into its site. In contrast, other features differ between the two tests. For example, shortening of the side chain (3,4-dihydroxy-benzylamine) considerably weakens the affinity without interfering with competitiveness. This can be understood if 3,4-dihydroxy-benzylamine binds to the same domains of DAT as dopamine, be it with lower affinity because the distance between the amine and its point of interaction on DAT is not optimal. In addition, β-hydroxylation (epinephrine, norepinephrine, synephrin), although not favorable to affinity, does not interfere with competitiveness. Clearly, the two tests measure different aspects of structure–activity relationships. It is possible to encounter compounds that have a low affinity for DAT and yet competitively interact with dopamine; conversely, it is possible to encounter compounds that bind to DAT with high affinity and do not interact with dopamine competitively. Examples of the latter are the blocker-type compounds, semi-rigid cocaine analogs COCA4A and COCA4B; the next most potent compounds with this profile are the amino naphthalenes and L-tyrosinol. To our knowledge, it is not known how well, if at all, these compounds are translocated.

Acknowledgments

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