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Pharmacological Heterogeneity of the Cloned and Native Human Dopamine Transporter: Disassociation of [^{3}H]WIN 35,428 and [^{3}H]GBR 12,935 Binding

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SUMMARY

Controversy exists as to whether the functional state of the dopamine (DA) transporter is identical to sites mediating the specific binding of selective DA transporter radioligands. Therefore, we compared the pharmacological profile of numerous dopamine transport substrates and inhibitors on [^{3}H]DA uptake with the binding of [^{3}H]WIN 35,428 and [^{3}H]GBR 12,935 to COS-7 cells transiently expressing the cloned human DA transporter. [^{3}H]DA uptake and [^{3}H]WIN 35,428 binding was specific, saturable, and to a single class of binding sites with an estimated K_m/V_{max} of ~2 μM and 6 pmol/min/10^5 cells for DA uptake and K_d/B_{max} values of ~10 nM and 113 fmol/10^5 cells for [^{3}H]WIN 35,428. [^{3}H]DA uptake was inhibited in a concentration-dependent and uniphasic manner by dopaminergic agents with an appropriate rank order of potency for the DA transporter. Although most uptake blockers inhibited [^{3}H]WIN 35,428 binding in a uniphasic manner, WIN 35,428, Lu 19,005, D-amphetamine, and DA clearly displayed the presence of both high and low affinity components. Comparison of the K_i values for the inhibition of [^{3}H]DA uptake with [^{3}H]WIN 35,428 binding reveals that, for uptake blockers and D-amphetamine, it is the high affinity component that shares pharmacological identity with effects on DA uptake (r = 0.9985), whereas for DA it is the low affinity site. In striking contrast, however, [^{3}H]GBR 12,935 binding to COS-7 cells could not be made to exhibit a pharmacological profile indicative of the DA transporter and suggests that the site regulating functional [^{3}H]DA uptake may not be identical with sites labeled by [^{3}H]GBR 12,935 in these cells. Moreover, these sites appear unrelated to those previously described in native membranes as "piperazine acceptor" or P450 proteins. Comparison of K_i values and rank order of potency for the inhibition of [^{3}H]WIN 35,428 or [^{3}H]GBR 12,935 binding to human caudate membranes reveals pharmacological homology, but not identity, with that of the cloned DA uptake process. Taken together, these data suggest that 1) [^{3}H]WIN 35,428 recognizes two sites of the DA transporter, of which only one appears to represent the functional state of the protein, and 2) [^{3}H]WIN 35,428 and [^{3}H]GBR 12,935 do not appear to bind the same functional form/state of the DA transporter. Whether the nonidentity of binding sites is a manifestation of some post-translational regulatory event (e.g., phosphorylation/accessory binding protein) or caused by the existence of multiple molecular forms of the DA transporter is currently unknown.

The neuronal DA transporter regulates synaptic DA concentrations by sodium-dependent reuptake of DA into presynaptic nerve terminals, thereby terminating dopaminergic neurotransmission (1, 2). DA uptake sites have been identified and studied by radioligand binding techniques using numerous compounds, with two of the most extensively characterized molecules being [^{3}H]WIN 35,428 and [^{3}H]GBR 12,935 (reviewed in 1, 3, 4). The evidence that these radiolabeled compounds bind to the DAT is based on the observations that: both drug binding and DA uptake are sodium-dependent (2, 4); the regional brain localization for radiolabeled uptake inhibitors is selective for areas displaying dopaminergic nerve terminals (1, 4, 5); and the rank order of potencies for the inhibition of radiolabeled ligand binding and DA uptake are reasonably well correlated (e.g., 6, 7). A number of pharmacological and molecular findings have led, however, to suggestions that DA transport sites labeled by ligands such as [^{3}H]WIN 35,428 and [^{3}H]GBR 12,935 may not be directly reflective of the functional DA uptake process, and

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ABBREVIATIONS: BSA, bovine serum albumin; BTCP, *N*-[1-(2-benzo(b)thiophenyl)cyclohexyl] piperidine; DA, dopamine; DAT, dopamine transporter; EDTA, ethylenediaminetetraacetic acid; GABA, γ-aminobutyric acid; GBR 12,909, 1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-(3-phenylpropyl) piperazine; GBR 12,935, 1-[2-diphenylmethoxy]ethyl]-4-(3-phenylpropyl)-piperazine; Lu 19,005, (±)-(*trans*)-3-(3′,4′-dichlorophenyl)-*N*-methyl-1-indanamine, indatraline; MPP$^+$,*N*-methyl-4-phenylpyridine; NE, norepinephrine; PMSF, phenylmethylsulphonyl fluoride; SDS, sodium dodecyl sulphate; SSC, standard saline citrate; WIN 35,428, 2β-carbomethoxy 3β-(4-fluorophenyl)tropane.

that [^{3}H]WIN 35,428 and [^{3}H]GBR 12,935 may not even be labeling identical sites/states of the DA transporter.

Evidence for the lack of identity between DA uptake and ligand binding is obtained from studies showing differential effects of cations (8–11), Concanavalin A (12), and alkylating reagents (12, 13) on DA uptake and ligand binding, and from experiments showing a series of BTCP homologs displaying differential selectivity for [^{3}H]BTCP/[^{3}H]cocaine binding sites and inhibition of DA uptake (14). Moreover, putative substrates for the DAT, such as DA and amphetamine, are 20- to 100-fold more potent at inhibiting [^{3}H]DA uptake than as inhibitors of ligand binding (6, 7, 15). In addition, studies on rat embryonic mesencephalic neuronal cultures show a dramatic dissociation of [^{3}H]WIN 35,428 binding and DA uptake (16).

Although a number of explanations may account for the lack of correspondence between DA uptake and ligand binding [e.g., reorientation of the membrane transporter from the inside to the outside (17) and the existence of distinct amino acid residues for DA uptake and binding (18)], additional pharmacological and behavioral evidence suggests that [^{3}H]WIN 35,428 and [^{3}H]GBR 12,935 bind to different states or subtypes of the DA transporter. These include: a) the reported lack of complete correspondence between the ability of GBR 12,935 to inhibit [^{3}H]WIN 35,428 binding to DA transporter sites and vice versa (7, 19); b) the existence of noncompetitive interactions between various ligands (20–23); c) autoradiographic studies depicting heterogeneous subregional [^{3}H]WIN 35,428 and [^{3}H]GBR 12,935 binding patterns in striatum of normal rats (24); and d) after chronic self administration of cocaine, the existence of differential and region specific compensatory increases of either [^{3}H]WIN 35,428 or [^{3}H]GBR 12,935 binding sites (24). Behavioral studies also suggest that DA uptake inhibitor binding sites may be different. For example, effects on DA-mediated behavior cannot be predicted by degree of occupancy of the DA transporter by inhibitors of DA uptake (25–27) and, although chronic treatment with cocaine results in tolerance and cross-tolerance to WIN 35,428, it does not to GBR 12,909 (28). Moreover, although GBR is more potent than cocaine at inhibiting DA uptake and WIN 35,428 binding in rat and monkey striatum, it is less potent than cocaine in locomotive responses (29).

Although the human, bovine, and rat DA transporters have been cloned (30–34), there have been no reports examining the identity of the DA uptake site/process with the binding characteristics and pharmacological profiles of both [^{3}H]WIN 35,428 and [^{3}H]GBR 12,935 in cells transfected with the cDNA encoding the human DAT.

In this communication we compare the pharmacological characteristics of [^{3}H]DA uptake, [^{3}H]WIN 35,428, and [^{3}H]GBR 12,935 binding in COS-7 cells expressing the cloned human DA transporter[1] to [^{3}H]WIN 35,428 and [^{3}H]GBR 12,935 binding patterns in normal human caudate. Although [^{3}H]WIN 35,428 has been shown to bind to two affinity states of both the cloned rat (35) and native monkey brain membrane DAT (7), we document that: 1) in addition to WIN 35,428, a number of compounds that are not cocaine congeners, including Lu 19,005, DA, and D-amphetamine, recognize two affinity components of the cloned human DAT, and that only one of these sites/states reflects the functional activity of the cloned DA uptake process; and 2) that [^{3}H]GBR 12,935 binding to the cloned DA transporter, under numerous experimental conditions, could not be made to exhibit a pharmacological profile indicative of the DA transporter despite the fact that both [^{3}H]WIN 35,428 and [^{3}H]GBR 12,935 binding in human brain membranes, although not identical, display pharmacological homology to the cloned human DA uptake site.

Materials and Methods

Materials. [^{3}H]DA (33.25 Ci/mmol), [^{3}H]GBR 12,935 (44.2 Ci/mmol), and [^{3}H]WIN 35,428 (83.4 Ci/mmol) were purchased from DuPont, New England Nuclear (Boston, MA). Amfonelic acid, Lu 19,005, and bupropion were from RBI. All other dopaminergic compounds were obtained from sources previously described (36). Autopsied human brains were obtained from four neurologically normal male subjects (mean age 57 ± 8 yrs; postmortem time 14 ± 2 hr).

Cloning and expression of the human DAT cDNA. An 800-bp Pst I-Sac I cDNA fragment subcloned from the bovine DAT (31) was used to probe a λgt10 human substantia nigra cDNA library (Clontech). Duplicate nylon filters (Hybond, Amersham) were prehybridized in 40% formamide, 50 mM Tris, pH 7.5, 0.1% sodium pyrophosphate, 0.2% BSA, 0.2% polyvinylpyrolidine 40,000, 0.2% ficoll 400,000, 1.1% SDS, 0.1% NaCl, 0.1 mg/ml sheared salmon sperm DNA at 42° for 5 hr, followed by hybridization overnight in a solution containing nick translated (Amersham) ^{32}P-labeled probe (8×10^5 dpm/ml). Filters were washed twice for 30 min with 2× SSC, 1% SDS at 50°. Phage DNA was purified from 11 positive plaques isolated after tertiary screening, inserts excised with *Eco*RI and subcloned into either pSP73 (Promega) or pBluescript (Stratagene). Two partial clones of 1.6 [clone D] and 1.2 kb [clone F] were used to construct a cDNA in pBluescript (pZDAT1) encoding the full length human DAT sequence. *Eco*RI and *Cla*I endonuclease digestion of clones D and F generated cDNA fragments of 1.3 kb and 0.7 kb, respectively, which after ligation were subcloned into the *Eco*RI multiple cloning site of pBluescript. The full length cDNA encoding the human DAT (pZDAT1) was sequenced in both directions using 7-deaza-GTP, Sequenase V2.0 (United States Biochemical), T3/T7 primers (Promega), as well as specific synthetic oligonucleotides (Biotechnology Service Centre, Hospital for Sick Children, Toronto) as internal primers.

An *Eco*RI fragment (~2 kb) excised from pZDAT1 was subcloned into the mammalian expression vector pcD (pcZDAT1) as previously described (37). For transient expression studies COS-7 cells were transfected with cesium chloride-purified pcZDAT1 by electroporation (40 μg of DNA/2.5×10^7 cells; 48 Ω, 135 mA, 500 μF), placed into 24-well plates, and cultured in Dulbecco's α-MEM supplemented with 10% fetal calf serum at 37°, 5% CO_2.

[^{3}H]DA Uptake. Measurement of DA uptake was performed on intact cells essentially as described by Giros *et al.* (30). Briefly, 2 to 4 days after transfection in 24-well plates (~2×10^5 cells seeded/well), medium was removed and wells were rinsed with 1.0 ml of uptake buffer (5 mM Tris, 7.5 mM HEPES, 120 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 mM ascorbic acid, 5 mM glucose, pH 7.1). Cells were preincubated, in duplicate, with the indicated concentrations of dopaminergic agents (10^{-13}-10^{-4} M) 5 min before the addition of [^{3}H]DA. Uptake was initiated by the addition of 0.5 ml of uptake buffer containing 20 nM [^{3}H]DA (final concentration, unless otherwise noted). After 5 min at 22°, wells were rinsed 2 times with 0.5 ml of uptake buffer, solubilized in 1% SDS (0.5 ml) for 15 min at 22°, and radioactivity incorporated into cells measured in a Beckman liquid scintillation counter (LS 6000SC).

[^{3}H]Ligand binding to cloned human DAT. [^{3}H]WIN 35,428 and [^{3}H]GBR 12,935 binding to COS-7 cells transfected with human DAT were measured under conditions similar to those described above. Briefly, medium was removed and cells were rinsed with 1.0 ml of uptake buffer. For saturation binding experiments cells were incubated,

[1] The sequence reported in this paper has been deposited in the Genbank database (accession number L24178).

in duplicate, with 1.0 ml of ice-cold uptake buffer containing increasing concentrations of [^{3}H]WIN 35,428 (0.125–100 nM final concentration) or [^{3}H]GBR 12,935 (0.2–20 nM). After 2 to 3 hr incubation at 4°, cells were washed 2 times with 1.0 ml of ice-cold buffer, solubilized in 1% SDS with bound radioligand measured by liquid scintillation counting as described above. Nonspecific binding for each radioligand was determined in the presence of 1 μM mazindol, GBR 12,909, and amfonelic acid. These values were taken directly from competition binding experiments (see below), which indicated that at a concentration of 1 μM the binding of [^{3}H]WIN 35,428 (~4 nM) was suppressed to 10% (mazindol or GBR 12,909) and 30% (amfonelic acid) of control. For [^{3}H]WIN 35,428 or [^{3}H]GBR 12,935 competition binding experiments, cells were incubated, in duplicate, with 1.0 ml of ice-cold uptake buffer containing ~4 nM (final concentration) of [^{3}H]WIN 35,428 or ~1 nM [^{3}H]GBR 12,935 and competing ligand (10^{-12}-10^{-4} M) for 2 to 3 hr at 4°. Assays were terminated, and bound radioactivity was measured as described above.

To directly compare the ability of dopaminergic uptake reagents to inhibit [^{3}H]DA uptake and radioligand binding, each compound/drug was tested on both [^{3}H]DA uptake and radioligand binding on the same day, using the same serial dilution of drug, and on the same batch of transfected cells.

[^{3}H]Ligand binding to human caudate membranes. Human caudate was homogenized in 100 volumes of the buffer described above or in sodium phosphate buffer [25 mM, pH 7.7, at 4°, final sodium concentration 48 mM] and centrifuged at 46,000 × *g* for 10 min. The resulting pellet was resuspended in 100 volumes of buffer, and the wash procedure was repeated. The membrane pellet was suspended to 5 mg (original wet weight)/ml and incubated (0.2 ml) in duplicate with either [^{3}H]GBR 12,935 (~1 nM) or [^{3}H]WIN 35,428 (~4 nM), and varying concentrations of competing ligands (10^{-13}-10^{-3} M) for 4 hr at 4° in a final vol of 0.6 ml. Incubation was terminated by rapid filtration of samples on glass fiber filtermats presoaked in 0.1% BSA using a Titertek cell harvester. Filters were washed twice with 5 ml of ice-cold buffer with radioactivity measured by LSC. As described above, each compound/drug was tested simultaneously on both [^{3}H]WIN 35,428 and [^{3}H]GBR 12,935 binding, using the same serial dilution of drug, and on the same human membrane preparation.

For comparative purposes, [^{3}H]GBR 12,935 (~1 nM) or [^{3}H]WIN 35,428 (~4 nM) binding were also assessed, using buffers and the filtration assay described above, to homogenized membrane preparations of transfected COS-7 cells and to detached intact whole cell suspensions collected after treatment of plated cells with 1× SSC for 20 min at 37°. Integrity of intact cell suspensions (>95%) were measured using trypan blue.

For some experiments, the binding characteristics of [^{3}H]WIN 35,428 (10 nM) and [^{3}H]GBR 12,935 (5 nM) to digitonin-solubilized human caudate and transfected COS-7 cell membranes were compared. Briefly, tissues (200 mg of caudate, or 5 × 10^7 transfected COS-7 cells) were homogenized as described above, and pellets were resuspended in buffer containing 1% digitonin for 1 hr at 4°, followed by centrifugation at 100,000 × *g* and assay for binding activity by Sephadex G-50 chromatography as previously described (36, 38).

Data analysis. Both saturation and ligand competition binding experiments were analyzed for one and two site fits using LIGAND as previously described (36, 38).

Results and Discussion

We have cloned a cDNA encoding a human dopamine transporter from a substantia nigral library. Nucleotide sequencing revealed an open reading frame of 1860 nucleotides encoding a protein of 620-amino acid residues in length with an estimated molecular mass of 68,424 Da in addition to 19 nucleotide residues 5′ to the putative initiation methionine and 130 nucleotides 3′ of the stop codon (data not shown). Hydropathic analysis of the deduced amino acid sequence of ZPDAT1 suggests the presence of 12 hydrophobic transmembrane regions, similar to the proposed transmembrane structure of the other members of the neurotransmitter transporter family (reviewed in 39). As shown in Fig. 1A, the nucleotide and deduced amino acid sequence reported here for the human DAT is identical to that reported by Vandenberg *et al.* (40), but differs in five nucleotide positions from the sequence previously reported by Giros *et al.* (30). Two of these differences result in amino acid substitutions, at residues 35 and 354 (M → K, C → S, respectively). The observed point mutations could be caused by the reported highly polymorphic nature of the human DAT (30, 40–42).

Comparisons of human DAT with dopamine transporters cloned from bovine (31) and rat (32–34) brain reveal 87 and 88% homology, respectively, at the nucleotide level, and 86 and 93% homology at the amino acid level. Among other members of the neurotransmitter transporter gene family, the dopamine transporter displays highest amino acid homology with the human noradrenaline (65%), serotonin (46%), and GABA (42%) transporters (43–45). Fig. 1B illustrates the proposed evolutionary relationships of the human DAT with other selected transporters of this gene family based on overall amino acid sequence homology. As recently reported (30, 40), the relatively large putative second extracellular loop of the human dopamine transporter contains three consensus sequences for N-linked glycosylation, compared to four for the rat and three for the bovine DAT, the functional significance of which is currently unknown (46). Potential sites for N-linked glycosylation appear conserved in the large second extracellular loop in similar positions in other members of the transporter family. Putative phosphorylation sites for PKA, PKC, and CaM kinase II are present in the proposed intracellular domains of the protein. Other structural features of the cloned human DA transporter have been recently reviewed elsewhere (39).

Dopamine uptake in COS-7 cells. [^{3}H]DA uptake in untransfected COS-7 cells or in mock-transfected cells was negligible (6% after 3 days) compared to cells transfected with pcZPDAT1, indicating the dependence of dopamine uptake on transfected pcZPDAT1. [^{3}H]DA uptake (20 nM) was found to be linear for approximately 15 min approaching a plateau by 3 hr (data not shown). Typically, at this concentration of [^{3}H]DA, total uptake counts were between 12,000 and 20,000 dpm/well and 600 and 1200 dpm/well for nonspecific binding (defined in the presence of 1 μM mazindol or GBR 12,909). As depicted in Fig. 2A, uptake of [^{3}H]DA in COS-7-transfected cells is concentration-dependent and saturable, with an estimated K_m of 2.4 ± 0.4 μM and V_{max} of 6.2 ± 0.4 pmol/min/10^5 cells as determined by Eadie-Hofstee analysis. The observed K_m value for [^{3}H]DA uptake is similar to that reported previously in various cell lines for the cloned human [1.2 μM], bovine [31.5 μM], and rat DATs [885–1190 nM] (see 30–34), although the K_m value of [^{3}H]DA uptake in either rat or human native synaptosome is approximately 10-fold lower (100–500 nM) (1, 11, 12, 47). Conceivably, differences in glycosylation patterns (46) and/or the presence of endogenous dopamine (6) may contribute to the overestimation of K_m values in native membranes. Interestingly enough, no such differences are observed for the NE, 5-HT, or GABA transporters (43, 48, 49).

[^{3}H]DA uptake was inhibited by all dopaminergic compounds tested in a concentration-dependent, stereoselective, and uniphasic manner with an appropriate rank order of potency: (+)

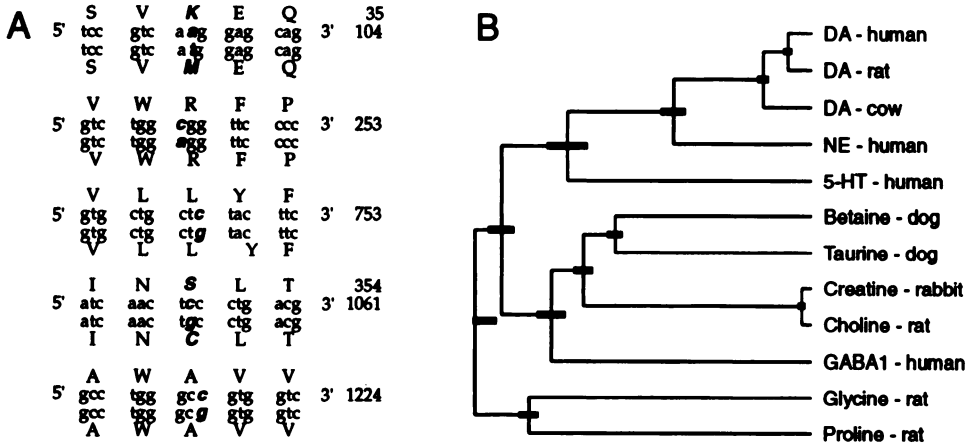

Fig. 1. A, Sequence divergence of the cloned human DATs: nucleotide (*lower case*) and deduced amino acid (*upper case*) sequences of the human dopamine transporter cDNAs in regions surrounding observed sequence differences (**bold face**). Upper sequence of pair, this paper, and (40); lower sequence of pair, (30). Numbers at right denote positions of variant nucleotide or amino acid residues (numbering begins at putative translational start site). B, Calculated evolutionary relationship of the human DAT with other selected members of transporter family based on overall amino acid sequence homology. Results are based on an UPGMA (unweighted pair group method with arithmetic mean) using a PAM-250 scoring matrix (63). Length of *horizontal lines* is proportional to the estimated evolutionary distance between sequences. ***Bold heavy lines*** indicate standard error of branch positions. (human dopamine, this paper; rat dopamine (32, 33, 34); bovine dopamine (31); human noradrenaline (43); human serotonin (44); canine betaine (64); canine taurine (65); rabbit creatine (66); rat choline (67); human GABA (45); rat glycine (68); rat proline (69)).

diclofensine ≈ amfonelic acid > Lu 19,005 ≈ WIN 35,428 ≈ GBR 12,909 > methylphenidate > nomifensine > mazindol > D-amphetamine > (−) diclofensine > cocaine ≈ bupropion > dopamine > norepinephrine > serotonin (see Fig. 2B for representative competition curves). K_i values for these compounds are listed in Table 1 along with respective Hill coefficients. Comparisons of the estimated K_i values of these compounds with those of the human DAT clone reported by Giros *et al.* (30) shows general agreement (<4-fold differences in K_i) with two major exceptions. One such difference is seen with D-amphetamine, which displays high affinity (116 nM) for [^{3}H]DA uptake, whereas Giros *et al.* (30) report a >10-fold lower affinity (2,260 nM). In rat striatum, dopamine uptake is inhibited by D-amphetamine with K_i values of 225 to 880 nM (15, 39), whereas in studies using rat and bovine clones K_i values of 880 to 2,160 nM and 386 nM (30–32) have been reported, respectively. The observed discrepancy in the effect of D-amphetamine on the two human cloned transporters cannot be ascribed, however, to either different methodologies or the presence of endogenous dopamine, because assay procedures for [^{3}H]DA uptake were identical with that of Giros *et al.* (30). It is possible that cell type differences or level of DAT expression are involved, because we are transiently expressing DAT in COS-7 cells, whereas Giros *et al.* (30) stably expressed DAT in Ltk$^-$ cells. This appears unlikely, however, because the same authors report a K_i value for D-amphetamine of ~880 nM for the rat DAT when transiently expressed in COS-7 cells (32), whereas a K_i for D-amphetamine (386 nM) more in line with our value is obtained from CV-1 cells expressing the bovine DAT (31). Similarly, a greater than 10-fold difference in the estimated K_i of cocaine for [^{3}H]DA uptake is seen between our cloned human DAT (~700 nM) and that of Giros *et al.* (58 nM) (30). The reported affinity of cocaine for DA uptake in native rat and human brain synaptosomes is ~600 nM (6, 50, 51). As described above, the observed amino acid residue differences, in particular C → S^{356}, between the two clones may be the molecular basis for the observed differences in ligand affinity. Site-directed mutagenesis studies on the rat cloned DAT suggest that serine residues in the seventh transmembrane region are differentially important for DA uptake and binding of cocaine (18).

[^{3}H]WIN 35,428 Binding. To assess whether the molecular form of DAT that mediates [^{3}H]DA uptake is identical to that recognized by various radioligands, we compared the pharmacological rank order of potency of a series of dopaminergic ligands in inhibiting [^{3}H]DA uptake with their ability to inhibit the binding of [^{3}H]WIN 35,428 to expressed DAT in COS-7 cells. As depicted in Fig. 3A, [^{3}H]WIN 35,428 binding to these cells is concentration-dependent, saturable, and of high affinity. Scatchard analysis indicates a single class of high affinity [^{3}H]WIN 35,428 binding sites with an estimated K_d of ~10.4 ± 0.6 nM (Fig. 3A, *inset*) and B_{max} of 113 ± 18 fmol/10^5 cells using 1 μM mazindol to determine nonspecific binding. Slight differences in both the estimated K_d and B_{max} of [^{3}H]WIN 35,428 binding were observed when 1 μM GBR 12,909 (K_d ~14 nM; B_{max} ~135 fmol/10^5 cells) or amfonelic acid (K_d ~8 nM; B_{max} ~88 fmol/10^5 cells) were used to define nonspecific binding (see below). These values are all within range of the reported K_d (5–16 nM) for [^{3}H]WIN 35,428 in native monkey and canine brain membranes (7, 52).

As illustrated in Fig. 3B, most dopaminergic uptake blockers and putative substrates inhibited [^{3}H]WIN 35,428 binding (~4 nM; representing total bound counts of 8–12,000 dpm/well and 600–1,100 dpm/well for nonspecific) in a concentration-dependent and uniphasic manner. Notable exceptions, as illustrated in Fig. 3C, were Lu 19,005, WIN 35,428, dopamine, and D-amphetamine, in which [^{3}H]WIN 35,428 competition curves were clearly biphasic, displaying Hill coefficients considerably less than unity, and which could be resolved, by computer assisted analysis, into two components of high and low affinity. K_i values and Hill coefficients for these and other dopaminergic agents along with the proportion of [^{3}H]WIN 35,428 binding sites recognized by various compounds as existing in either a high or low affinity form are listed in Table 1. The rank order of potency of numerous DA uptake inhibitors and substrates for the inhibition of [^{3}H]WIN 35,428 binding to transfected

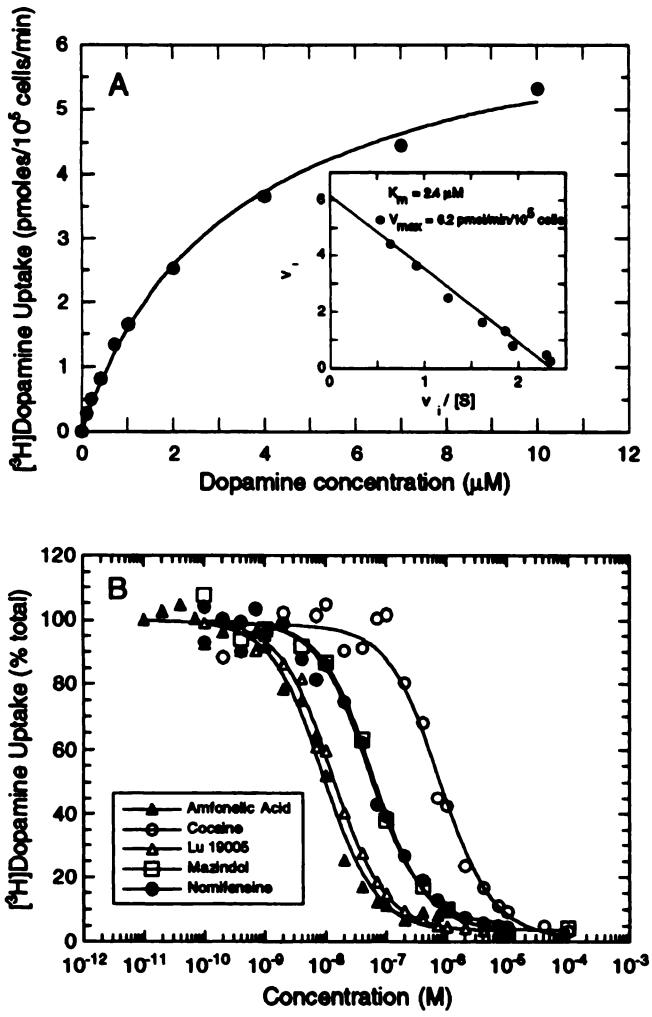

Fig. 2. Characterization of [^{3}H]DA uptake in transfected COS-7 cells: A, Saturation isotherm for [^{3}H]DA uptake in COS-7 cells after transfection with human DA transporter cDNA. Cells (~2.5 × 10^6) were incubated with [^{3}H]DA (20 nM) and increasing concentrations of unlabeled DA as described in Materials and Methods. *Inset*, Eadie-Hofstee transformation of the same kinetic data. B, Pharmacological profile of [^{3}H]DA uptake. Cells (~2.5 × 10^5) were preincubated with various concentrations of DA uptake inhibitors (10^{-12}-10^{-4} M) followed by the addition of [^{3}H]DA (20 nM) for 5 min at 22° as described in Materials and Methods. All curves were best fitted to a single class of binding site. Results depicted are representative of two to four independent experiments each conducted in duplicate.

COS-7 cells is as follows: Lu 19,005 (high) > amfonelic acid ≈ GBR 12,909 > (+) diclofensine > WIN 35,428 (high) > methylphenidate ≈ mazindol > nomifensine > dopamine (high) > D-amphetamine (high) > cocaine ≈ (−) diclofensine > Lu 19,005 (low) ≈ bupropion > WIN 35,428 (low) > dopamine (low) > noradrenaline > D-amphetamine (low) > serotonin.

As outlined in Table 1, the proportion of [^{3}H]WIN 35,428 binding components recognized by DA uptake blockers, WIN 35,428, or Lu 19,005, as existing in either high or low affinity were ~75%/25%, whereas for putative substrates of DA transport, dopamine and D-amphetamine, the proportion of high and low affinity [^{3}H]WIN 35,428 binding sites were equally distributed at ~50%. The proportion of observed high and low affinity binding components for both WIN 35,428 and Lu 19,005 do not appear to correlate well with the two [^{3}H]WIN

TABLE 1

K_i values for inhibition of [^{3}H]DA uptake and [^{3}H]WIN 35,428 binding to the cloned human DAT expressed in COS-7 cells

K_i values for the inhibition of [^{3}H]DA uptake and [^{3}H]WIN 35,428 binding for various compounds to transfected COS-7 cells are listed in accordance to their rank order of potency for the [^{3}H]DA uptake process. Where biphasic competition curves were observed, K_i values and approximate proportions of both high (H) and low (L) affinity components, as estimated by computer assisted analysis, are given. Data represent the means of two to four independent experiments, each conducted in duplicate, with less than 15% variation.

Compound	[^{3}H]DA Uptake		[^{3}H]WIN 35,428 Binding		
	K_i	Hill coefficient	K_i		Hill coefficient
	nM		nM		
(+) Diclofensine	8.0	1.00	4.6		0.91
Amfonelic acid	9.1	0.99	3.7[a]		0.97
Lu 19,005	13	1.01	3.2	(H 80%)	0.37
			890	(L 20%)	
WIN 35,428	13	0.99	10	(H 70%)	0.34
			1,900	(L 30%)	
GBR 12,909	14	1.06	3.8		1.00
Methylphenidate	42	0.92	21		0.86
Nomifensine	53	0.97	42		1.00
Mazindol	60	0.94	24		0.94
D-Amphetamine	116	0.92	190	(H 41%)	0.46
			21,000	(L 59%)	
(−) Diclofensine	606	0.99	240		1.00
Cocaine	743	0.99	240		0.97
Bupropion	784	0.93	950		0.92
Dopamine	1,770	1.00	67	(H 51%)	0.38
			3,100	(L 49%)	
Noradrenaline	8,500		9,800		
Serotonin	44,000		110,000		
Reserpine	>10,000		>10,000		
Ketanserin	>10,000		>10,000		
Budipine	>100,000		>100,000		

[a] Represents ~70% inhibition of [^{3}H]WIN 35,428 binding.

35,428 binding sites (15%/85%) (7) or [^{3}H]cocaine binding sites (6%/94%) (53) reported in native monkey membranes, or with the proportions of high and low affinity WIN 35,428 binding components (3%/97%) in native (54) or cloned rat DAT expressed in COS-7 cells (35). Moreover, some reports suggest the existence of only a single component for WIN 35,428 in some instances (18, 54). Although the proportions of these sites may differ in various tissues, all reports of the estimated affinity of WIN 35,428 for the high affinity site (~5 nM) are remarkably consistent.

As depicted in Fig. 3B, of all the compounds tested, only amfonelic acid does not appear to recognize binding components that appear to represent low affinity for the DA uptake blockers WIN 35,428 and Lu 19,005 (~20–30% of the total [^{3}H]WIN 35,428 bound). The inability of amfonelic acid to displace [^{3}H]WIN 35,428 binding to these sites at concentrations up to 10 μM seems to account for the ~30% reduction in estimated B_{max} of [^{3}H]WIN 35,428 binding when it was used to define nonspecific binding (see above). The molecular basis for the incomplete inhibition profiles by amfonelic acid is unknown, particularly in light of the observation that all compounds inhibited [^{3}H]DA uptake to the same baseline levels (see fig. 2B and Table 1). In any event, this is the first report depicting: 1) the existence of high and low affinity [^{3}H]WIN 35,428 components for the cloned human DAT that are recognized by compounds other than WIN 35,428 itself to include Lu 19,005, dopamine, and D-amphetamine; and 2) that the proportion of [^{3}H]WIN 35,428 sites recognized as either high or low affinity differs for DA uptake blockers and putative substrates.

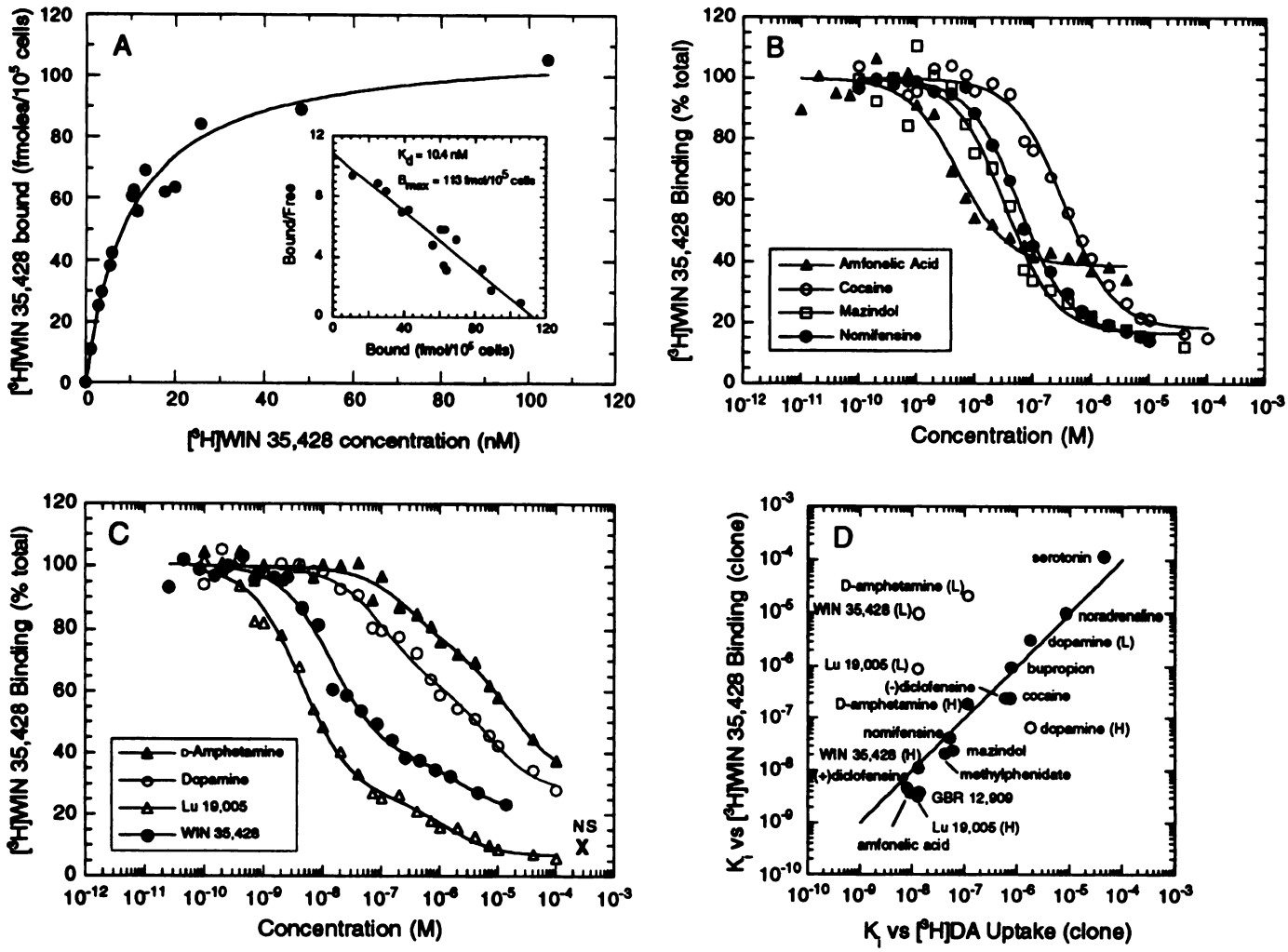

Fig. 3. Characterization of [³H]WIN 35,428 binding in transfected COS-7 cells. A, Saturation isotherm for [³H]WIN 35,428 binding. Cells (~2.5 × 10^5) were incubated with increasing concentrations of [³H]WIN 35,428 (0.12–100 nM) for 2 to 3 hr at 4° and assayed for binding activity as described in Materials and Methods. Nonspecific binding (8–10% of total binding) was assessed in the presence of 1 μM mazindol. *Inset*, Scatchard transformation of same data. B, Pharmacological profile of selected compounds to inhibit [³H]WIN 35,428 binding in COS-7 cells after transfection with human DA transporter cDNA. Cells were incubated with [³H]WIN 35,428 (4–5 nM) and with the indicated concentrations of putative uptake inhibitors for 2 to 3 hr at 4° as described in Materials and Methods. Nonspecific binding was defined using 1 μM mazindol. Data were best fit to a single class of binding site. C, [³H]WIN 35,428 competition curves with DAT ligands WIN 35,428, DA, D-amphetamine, and Lu 19,005, which could best be resolved into two affinity components as determined by computer assisted analysis using LIGAND. Assay conditions same as in *B*. Results shown are representative curves of 2 to 4 independent experiments, each conducted in duplicate. K_i values and corresponding Hill coefficients are listed in Table 1. NS, nonspecific binding determined in the presence of 1 μM mazindol. D, Correlational plot between the K_i values of DA uptake inhibitors and putative substrates on [³H]DA uptake with [³H]WIN 35,428 binding to the cloned human dopamine transporter. Line of identity is indicated. H (high affinity), L (low affinity).

Whether the same phenomenon can be seen in other cell lines expressing the human DAT remains to be determined.

As shown in Fig. 3D, estimated K_i values for the inhibition of [³H]DA uptake by a series of drugs are highly correlated to values obtained for the inhibition of [³H]WIN 35,428 binding with a virtual one-to-one correspondence in affinities. Comparison of the K_i values for the inhibition of [³H]DA uptake to [³H]WIN 35,428 binding for those compounds exhibiting biphasic displacement patterns reveals that for WIN 35,428, Lu 19,005, and D-amphetamine it is the high affinity component that shares pharmacological identity with effects on cloned [³H]DA uptake, whereas for dopamine it is the low affinity site. Interestingly enough, the K_i of dopamine at the high affinity [³H]WIN 35,428 binding site (~100 nM) of the cloned transporter agrees exceptionally well with that of DA uptake in native human brain synaptosomes (~200 nM), but not with the K_m for DA uptake by cloned DAT reported here (~2 μM) or by others (see above). Although the functional significance is currently unknown, the recognition of two affinity sites of [³H]WIN 35,428 binding by D-amphetamine, dopamine, Lu 19,005, and WIN 35,428 suggests that either [³H]WIN 35,428 is binding to more than one site on the transporter, or it is binding to different conformational states of the protein of which only one is functionally correlated with that of cloned DA uptake process.

[³H]GBR 12,935 Binding in COS-7 cells. Unlike the results obtained with [³H]WIN 35,428, the binding of [³H]GBR 12,935 to COS-7 cells transfected with the human DAT was not saturable and was linear up to 22 nM. These results were completely unexpected considering that the observed K_d for

[^{3}H]GBR 12,935 binding in human caudate is 1 to 3.5 nM (1, 55) and that unlabeled GBR 12,909 completely inhibited both [^{3}H]DA uptake and [^{3}H]WIN 35,428 binding in these cells with high affinity (Fig. 2B, Table 1). As illustrated in Fig. 4A, whereas [^{3}H]GBR 12,935 did appear to bind to sites over and above that seen with mock transfected cells (30,000 dpm/well versus 12,000 dpm/well, respectively) the binding was not inhibited by the presence of high concentrations of numerous dopamine uptake inhibitors, with the exception of GBR 12,909 (1 μM) and the inactive isomer of DA uptake, (−) diclofensine (100 μM), each inhibiting ~50% of the total [^{3}H]GBR 12,935 bound. Displacement of [^{3}H]GBR 12,935 by these compounds was unrelated to effects on the DA transporter because, as depicted in Fig. 4B, similar levels of inhibition (50%) of [^{3}H] GBR 12,935 binding were observed in mock-transfected COS-7 cells. Moreover, displacement of [^{3}H]GBR 12,935 binding was not a temperature-dependent process, because identical observations were made when the binding assay was carried out at 22° instead of the standard 0 to 4°. In addition, procedures that increase signal/noise ratios for [^{3}H]GBR 12,935 binding, such as the presence of 0.1% BSA in the wash buffer, also had no effect. More importantly, the order of addition of specific inhibitors with the radioligand was not a factor, because preincubation of cells for 30 to 60 min with high concentrations of uptake inhibitors (10 μM) before the addition of 1 nM [^{3}H] GBR 12,935 still did not yield a specific binding signal (data not shown). Budipine, an inhibitor of cytochrome P-450IID1, which is responsible for a significant amount of the total binding of [^{3}H]GBR 12,935 in canine and human tissues (e.g., 36, 56), had no effect on [^{3}H]GBR 12,935 binding (or for that matter on dopamine uptake or [^{3}H]WIN 35,428 binding) at a concentration of 10 μM. These data suggest that the nonspecific binding of [^{3}H]GBR 12,935 to transfected COS-7 cells was not a result of the presence of, or interference by, endogenous cytochrome P-450/piperazine acceptor sites (36).

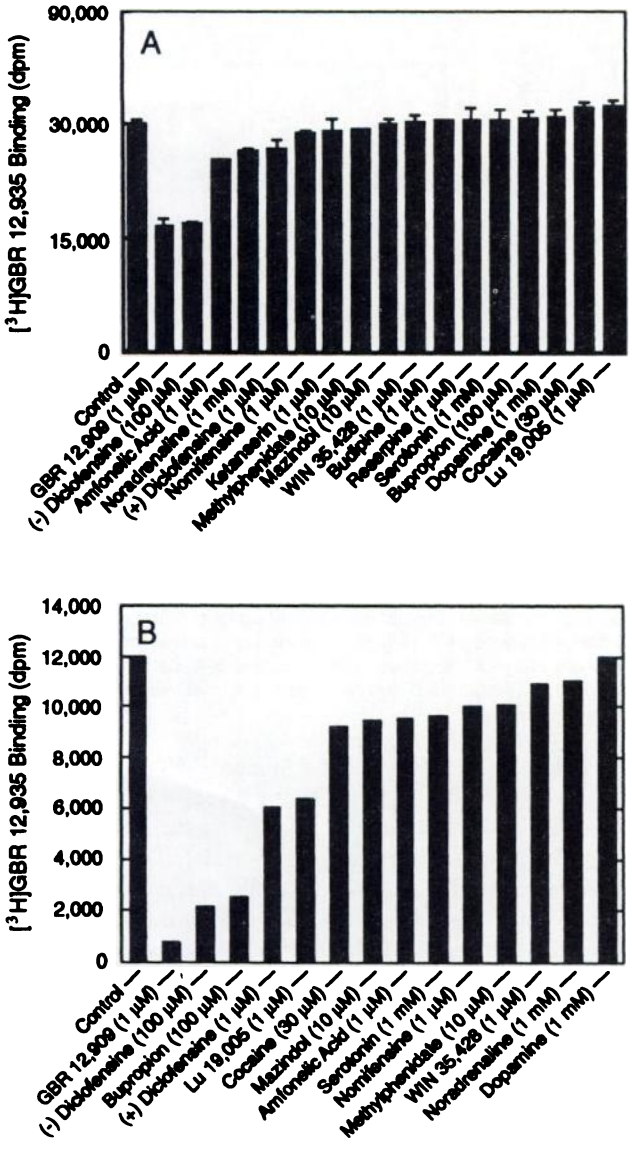

Fig. 4. Effects of DA uptake inhibitors and other compounds on the binding of [^{3}H]GBR 12,935 to A, transfected COS-7 cells and B, mock transfected cells: Cells (~2.5 × 10^5) were incubated with 1 nM [^{3}H]GBR 12,935 for 2 to 3 hr at 4° and assayed for binding activity as described in Materials and Methods. Concentrations of dopamine uptake inhibitors chosen are ≥100 times the estimated K_i determined against [^{3}H]DA uptake.

TABLE 2

K_i values for inhibition of [^{3}H]WIN 35,428 and [^{3}H]GBR 12,935 binding to human caudate membranes

K_i values for the inhibition of [^{3}H]WIN 35,428 and [^{3}H]GBR 12,935 binding for various compounds to human caudate membranes are listed in accordance to their rank order of potency for [^{3}H]WIN 35,428. Data represent the means of four independent experiments, each conducted in duplicate, with a standard error of the mean of less than 20%. Although most [^{3}H]GBR 12,935 competition curves were biphasic, only the estimated K_i values and corresponding Hill coefficients for the high affinity site are listed.

Compound	[^{3}H]WIN 35,428 Binding		[^{3}H]GBR 12,935 Binding	
	K_i	Hill coefficient	K_i	Hill coefficient
	nM		nM	
Lu 19,005	0.41	1.06	1.0	1.02
WIN 35,428	10.4	0.82	13.4	0.93
Mazindol	15.4	0.86	22.6	0.92
GBR 12,909	30.6	0.85	1.4	0.87
Nomifensine	40.2	0.93	56.4	0.98
Methylphenidate	61.0	0.98	50	0.91
Cocaine	68.5	0.91	331	0.97
Amfonelic acid	210	0.86	74.3	0.94
Bupropion	560	0.92	1,800	0.82
Dopamine	5,900	1.06	5,300	0.99
Noradrenaline	>10,000	0.91	>10,000	1.09
Serotonin	>100,000	0.90	>100,000	1.02

To test the possibility that the [^{3}H]GBR 12,935 binding site was more proteolytically sensitive (55) than either the [^{3}H] WIN 35,428 binding site or the DA uptake process, the pharmacological specificity of [^{3}H]GBR 12,935 binding was assessed in the presence of various protease inhibitors, including EDTA (100 μM), leupeptin (0.5 μg/ml), pepstatin (0.7 μg/ml), and PMSF (200 μM). Although these agents did not appear to affect either [^{3}H]DA uptake or [^{3}H]WIN 35,428 binding, no specific or pharmacologically relevant [^{3}H]GBR 12,935 binding site was observed (data not shown).

To ascertain the possibility that the lack of specific [^{3}H]GBR 12,935 binding was not simply caused by passive diffusion/membrane trapping and/or internalization of the radioligand into transfected cells, we permeabilized DAT-transfected COS-7 cells with digitonin. Treatment of cells with 0.003% digitonin for 6 min at 22° rendered >95% of cells trypan blue-sensitive. Although treated cells displayed a >93% loss of [^{3}H]DA uptake, and retained the ability to bind [^{3}H]WIN 35,428, no DAT-

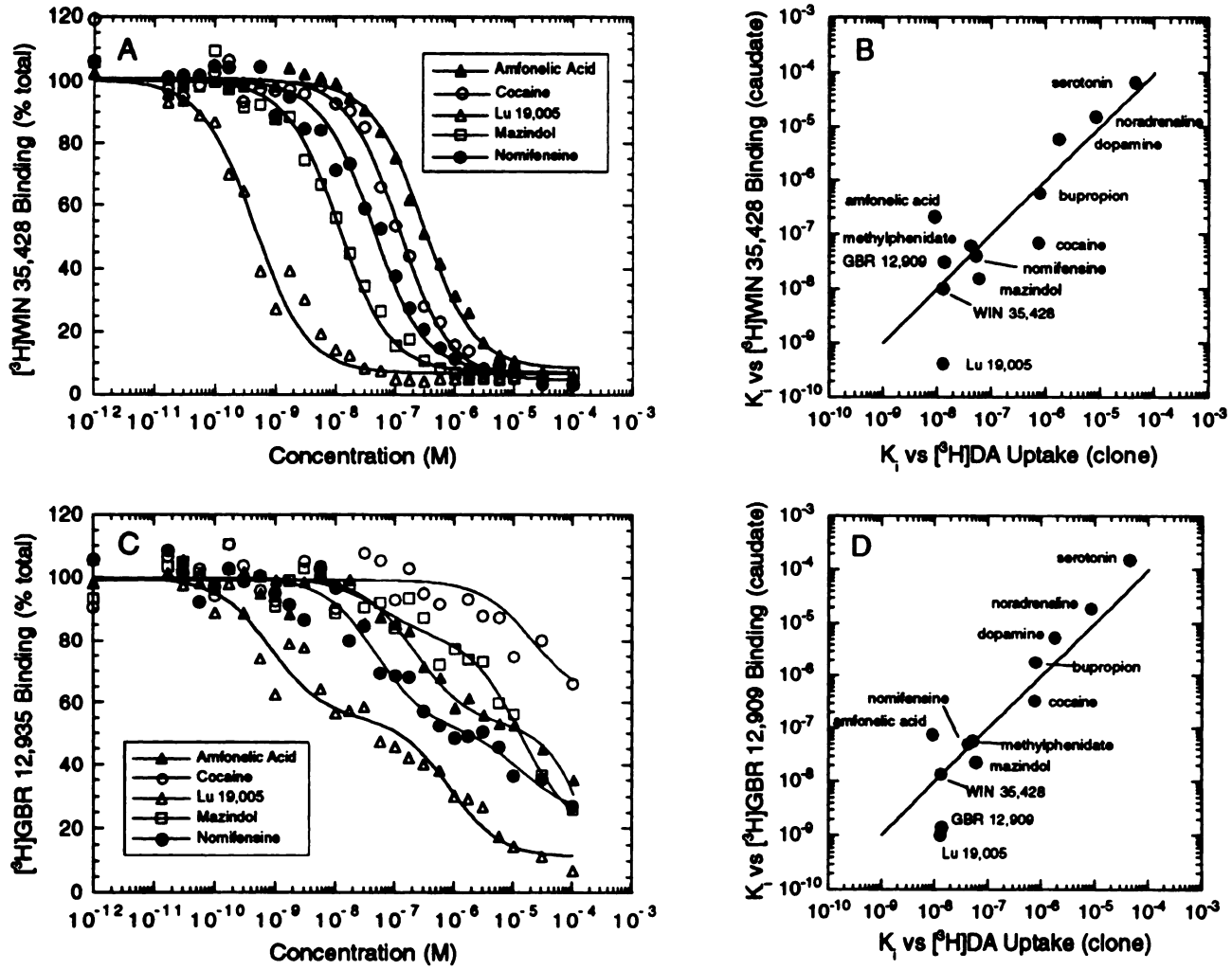

Fig. 5. Pharmacological profile of the abilities of selected DA uptake compounds to inhibit: A, [^{3}H]WIN 35,428 binding, and C, [^{3}H]GBR 12,935 binding to human caudate membranes. Correlational plots between the K_i values of DA uptake inhibitors and putative substrates on [^{3}H]DA uptake with B, [^{3}H]WIN 35,428 binding, and D, [^{3}H]GBR 12,935 binding to human caudate membranes. Membranes (~100 μg of protein) were incubated with either [^{3}H]WIN 35,428 (4 nM) or [^{3}H]GBR 12,935 (1 nM) with the indicated concentrations of DA uptake blockers and substrates for 2 to 3 hr at 4° and assayed for binding activity as described in Materials and Methods. Curves are representative of four independent determinations, each conducted in duplicate. All data were analyzed for one and two site fits as described. K_i values and corresponding Hill coefficients for these and other compounds are listed in Table 2.

relevant specific [^{3}H]GBR 12,935 binding could be detected (data not shown). Similarly, digitonin solubilization of COS-7 cells, [under conditions where appropriate DAT pharmacology for [^{3}H]GBR 12,935 or [^{3}H]WIN 35,428 binding can be obtained from solubilized human striatum after separation of bound from free radioligand by Sephadex G-50 column chromatography (36, 38)] failed to reveal specific, pharmacologically relevant binding sites for [^{3}H]GBR 12,935. These data suggest that [^{3}H]GBR 12,935 does not recognize the cloned human DAT even when given access to hydrophobic and appropriate cytosolic components of cells. Moreover, the inability of [^{3}H]GBR 12,935 to recognize cloned human DAT is not specific to COS-7 cells, because preliminary data from our laboratory[2] suggests that in Y1 cells (57) stably expressing the human DAT, no specific [^{3}H]GBR 12,935 binding can be detected, whereas both [^{3}H]DA uptake and [^{3}H]WIN 35,428 binding activities are clearly expressed. The inability to observe GBR binding appears not to be restricted to the human DAT cDNA because Patel *et al.* (46) recently noted that photoaffinity labeling and the specific incorporation of [^{125}I]DEEP, a structural analog of GBR 12,935, into a polypeptide of Mr ~100 kDa of the cloned rat DAT expressed in COS-7 cells could not be antagonized by high concentrations of cocaine. The above data raise the possibility that two distinct, but pharmacologically similar, DATs exist *in vivo*, of which only one specifically expresses [^{3}H]GBR 12,935 binding activity. Alternatively, some post-translational regulatory process, such as protein phosphorylation/dephosphorylation (58) or the requirement for an accessory binding protein, whose activity would be expected to be co-expressed in substantia nigral neurons *in vivo*, is required to enable DAT to bind [^{3}H]GBR 12,935.

To date, there have been no reports examining the pharmacological profiles of [^{3}H]WIN 35,428 and [^{3}H]GBR 12,935 binding to native human membranes under identical assay conditions. To assess whether pharmacologically distinct human DATs are recognized by these ligands, the rank order of

[2] Z. B. Pristupa and H. B. Niznik, unpublished observations.

potency for various dopamine uptake inhibitors and putative substrates on the binding of either [^{3}H]WIN 35,428 (~4 nM) and [^{3}H]GBR 12,935 (~1 nM) to human caudate membranes was determined. As depicted in Fig. 5A, all compounds tested inhibited [^{3}H]WIN 35,428 binding in a uniphasic manner and with a pharmacological profile clearly suggestive of the DA transporter. K_i values and corresponding Hill coefficients for these compounds are listed in Table 2. Competition of [^{3}H] WIN 35,428 by unlabeled WIN 35,428 (K_d ~10 nM) in three out of four of the human brain membranes tested could not be resolved into high and low affinity components. In one brain, however, the proportion of high and low affinity WIN 35,428 binding components was 25%/75%, as has been reported by others (7). As depicted in Fig. 5B comparison of the K_i values for the inhibition of [^{3}H]WIN 35,428 binding to human brain membranes with those obtained on [^{3}H]DA uptake in COS-7 cells reveals strong pharmacological homology. Several compounds, however, such as amfonelic acid and Lu 19,005, display significantly divergent affinities (10- to 50-fold). Moreover, the rank order of potency for these compounds, although clearly indicative of a dopamine transporter, does not identically match that observed for the cloned DAT (Fig. 3A). Although [^{3}H] WIN 35,428 binding parameters for the cloned human DAT were assessed mainly on plated COS-7 cells, whereas those of [^{3}H]WIN 35,428 binding in brain were determined on homogenized tissues, only minor differences in the estimated K_i values were seen in intact-whole cell versus homogenized COS-7 cell preparations for compounds such as methylphenidate (K_i ~21 versus ~37 nM), mazindol (K_i ~24 versus ~13 nM), and WIN 35,428 (K_i ~10 versus ~31 nM) respectively. Under the same conditions, these compounds expressed affinities for [^{3}H]GBR 12,935 binding of >10 μM (data not shown). It should be noted that transfected COS-7 cells, once lifted from plates, display a substantial loss (~80%) of specific [^{3}H]WIN 35,428 binding, thus precluding extensive pharmacological characterizations. Although the molecular mechanism, if any, is unknown, it would be of interest to determine whether similar observations are seen with other cloned transporters and other cell lines.

As depicted in Fig. 5C, [^{3}H]GBR 12,935 binding to membranes from human caudate was inhibited by numerous DA uptake agents with characteristics similar to those reported by other laboratories (6, 15, 38, 55, 59). Most drugs only inhibited a portion (~45%) of the total [^{3}H]GBR 12,935 bound. The proportions of the high affinity [^{3}H]GBR 12,935 binding component was variable among the brain samples examined and ranged from 30 to 60% of total binding. It is this site that displays a pharmacological profile suggestive of the DA transporter. The remaining sites are bound by some compounds (e.g., GBR 12,909, Lu 19,005, WIN 35,428, and mazindol) with poor affinity. K_i values for all compounds at the high affinity [^{3}H] GBR 12,935 binding site are listed in Table 2. As depicted in Fig. 5D, comparison of these values with those obtained on DA uptake by COS-7 cells clearly reveals a profile correlative with a DA transporter, but not identical/homologous to the rank order reported for either [^{3}H]DA uptake (Fig. 3D) or [^{3}H]WIN 35,428 binding (Fig. 3D, Fig. 5B). As can be seen from these figures and Table 2, compared to values obtained for the cloned transporter, several outliers were found, including GBR 12,909, Lu 19,005, and amfonelic acid. Taken together, these data provide some evidence for the existence of pharmacologically overlapping [^{3}H]WIN 35,428- and [^{3}H]GBR 12,935-labeled DATs in native membrane preparations, as suggested recently (3).

Although the available data cannot clearly account for the dissociation of [^{3}H]WIN 35,428 and [^{3}H]GBR 12,935 binding sites of the cloned DAT, we suggest that the lack of correspondence is not a product of distinct genes, but rather, is because of the existence of particular conformational states of the DAT allowing for the expression of high affinity [^{3}H]WIN 35,428 or [^{3}H]GBR 12,935 sensitive sites. We predict, as well, that intermediate states of the DAT protein will allow for both [^{3}H]WIN 35,428 and [^{3}H]GBR 12,935 binding to a single polypeptide. The molecular mechanisms that may be involved in the maintenance of either [^{3}H]WIN 35,428- or [^{3}H]GBR 12,935-sensitive components are unknown at present, although a number of possibilities, including protein phosphorylation and corresponding changes in charge density, were mentioned above (58). Evidence that [^{3}H]WIN 35,428 and [^{3}H]GBR 12,935 binding components appear not to be products of distinct genes may be derived from recent immunoprecipitation studies of photaffinity-labeled rat brain membranes (60). Antibodies raised against peptide sequences of the C-terminal portion of the second large extracellular loop of the rat DAT appeared to immunoprecipitate DATs photaffinity-labeled with either GBR or cocaine analogs, [^{125}I]DEEP and [^{125}I]RTI-82, respectively. If [^{3}H]WIN 35,428/cocaine and [^{3}H]GBR 12,935 binding sites were truly distinct, possibly reflecting the activity of different genes, then antibodies generated to a fairly variable region of cloned transporters would not be expected to immunoprecipitate both polypeptides. Similarly, the ability of unlabeled GBR 12,909, a close structural analog of [^{3}H]GBR 12,935, to inhibit both [^{3}H]DA uptake and [^{3}H]WIN 35,428 binding to the cloned DAT suggests that one polypeptide confers sensitivity to both ligands. The existence of mRNA splice variants for human or rat DATs may account for the possibility of heterogeneous populations of DATs in native tissues but, unlike the glycine transporter (61), these have not been observed. Whatever the mechanism, it is clear that the binding characteristics of [^{3}H] WIN 35,428 or [^{3}H]GBR 12,935 have not always consistently yielded identical results when assessing the preponderance of DATs in numerous experimental treatment paradigms (e.g., chronic cocaine or drug treatment) and neuropathological states (e.g., Parkinson's disease) (see 24, 62 and references cited therein). Further work will be necessary to come to a complete understanding of those molecular mechanisms regulating the structure and function of DAT in the maintenance of [^{3}H]WIN 35,428 and [^{3}H]GBR 12,935 binding activity.

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