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ACCELERATED COMMUNICATION

Cloning, Pharmacological Characterization, and Chromosome Assignment of the Human Dopamine Transporter

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SUMMARY

We have screened a human substantia nigra cDNA library with probes derived from the rat dopamine transporter. A 3.5-kilobase cDNA clone was isolated and its corresponding gene was located on the distal end of chromosome 5 (5p15.3). This human clone codes for a 620-amino acid protein with a calculated molecular weight of 68,517. Hydropathicity analysis suggests the presence of 12 putative transmembrane domains, a characteristic feature of sodium-dependent neurotransmitter carriers. The rat and the human dopamine transporters are 92% homologous. When permanently expressed in mouse fibroblast Ltk$^-$ cells, the human clone is able to induce a saturable, time- and sodium-dependent, dopamine uptake. This transport is blocked by psychostimulant drugs (cocaine, *l*- and *d*-amphetamine, and phencyclidine), neurotoxins (6-hydroxydopamine and *N*-methyl-4-phenylpyridine (MPP)$^+$), neurotransmitters (epinephrine, norepinephrine, γ-aminobutyric acid, and serotonin), antidepressants (amitriptyline, bupropion, desipramine, mazindol, nomifensine, and nortriptyline), and various uptake inhibitors (mazindol, GBR 12783, GBR 12909, and amfonelic acid). The rank orders of the K_i values of these substances at the human and the rat dopamine transporters are highly correlated ($r = 0.998$). The cloning of DNA human dopamine transporter gene has allowed establishment of a cell line stably expressing the human dopamine transporter and, for the first time, an extensive characterization of its pharmacology. Furthermore, these newly developed tools will help in the study of the regulation of dopamine transport in humans and in the clarification of the potential role of the dopamine transporter in a variety of disease states.

The monoamine DA plays a key role in the regulation of various CNS functions (locomotor activity, positive reinforcement, spatial memory, higher cognitive functions, and neurohormone release) and in major neurological diseases, as well as psychiatric disorders (Parkinson's disease, Tourette syndrome, schizophrenia, and bipolar affective disorder). DA mediates such a variety of functions through the stimulation of at least five distinct subtypes of guanine nucleotide-binding protein-coupled receptors (1, 2). Subtypes of these receptors have been shown to be the target of neuroleptic drugs and, thus, could be involved in the etiology of schizophrenia (3, 4). Various subtypes of DA receptors may act in concert (5) to mediate the neurotransmitter role of DA in the CNS.

Therefore, the acute or chronic concentration of DA in the CNS has crucial functional consequences. It is well established that the concentration of DA is acutely controlled in the intercellular space mostly by reuptake mechanisms (6). After its release, the concentration of DA at or around the synapse is rapidly reduced, due to the presence of uptake sites on the plasma membrane of presynaptic dopaminergic nerve endings. Consequently, the DAT plays a pivotal role in the regulation of pre- and postsynaptic dopaminergic transmission.

The DA uptake site, aside from its physiological role, is also the target of a variety of psychoactive agents, such as cocaine, amphetamine, and PCP derivatives (7). It has also been hypothesized that neurotoxins like 6-OH-DA and MPP$^+$ (the active derivative of MPTP) enter dopaminergic neurons by the route of the reuptake site (8). In the case of human Parkinson's disease, the severe depletion of striatal DA is overcome by the administration of L-DOPA to the patients (9). Here again, it is generally believed that L-DOPA penetrates residual dopami-

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ABBREVIATIONS: DA, dopamine; DAT, dopamine transporter; SSC, standard saline citrate; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PKC, protein kinase C; CNS, central nervous system; PCP, phencyclidine; PCR, polymerase chain reaction; Kb, kilobases; 6-OH-DA, 6-hydroxydopamine; GABA, γ-aminobutyric acid; NA, norepinephrine; TM, transmembrane; PKA, cAMP-dependent protein kinase; NAT, noradrenaline transporter; DOPA, 3,4-dihydroxyphenylalanine; MPP$^+$, *N*-methyl-4-phenylpyridine; MPTP, *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

nergic neurons and is subsequently decarboxylated into DA. However, it is not known whether, in the human brain, this uptake process involves the plasma membrane DAT. The implication of DA in the etiology of some aspects of depression (locomotor impairment, emotional withdrawal), although not clearly proven, has long been suspected (10). This is further illustrated by the high incidence of depression in Parkinsonian patients and the antidepressant action of L-DOPA. Thus, these data, although sometimes controversial, strongly suggest that the DAT not only plays a pivotal role at the synapse but also appears to be a critical target for substances of abuse, as well as drugs widely used in the clinic. In the past the regulation of DA transport in the animal brain has been intensively studied, but a good experimental model for human DA transport has so far been missing.

The neurotransmitter reuptake process is driven by the energy of the sodium gradient generated by the Na^+,K^+-ATPase (11). The uptake is mediated by proteins that co-transport neurotransmitters and ions. Recently, cDNAs encoding the rat and human GABA transporter, as well as the human NA uptake sites, have been cloned (12–14). They all share significant levels of homology, thus defining a new family of transporters. Taking advantage of this information, we and others have cloned the cDNA coding for the rat and the bovine DA and serotonin reuptake site (15–20). We now report the cloning of the DAT from a human substantia nigra cDNA library. The isolation of a cDNA and the establishment of a clonal cell line expressing the human DAT have allowed the determination, for the first time, of the detailed pharmacology of the human brain DAT.

Experimental Procedures

PCR amplification. Two cDNA probes were obtained by PCR amplification of the rat DAT (17). Probe A, from nucleotides 109 to 796 of the rat sequence, was obtained with the primers 5′-CAGAACGGAGTGCAGCTG-3′ (nucleotides 109–126) and 5′-ACACCACCTTCCCTGAGGT-3′ (nucleotides 796–778). Probe B, amplified with the primers 5′-CTGGCTACTTTCCTGCTGTCTCT-3′ (nucleotides 1357–1379) and 5′-TGGCAGCTGTCTCCTTCCACTTTA-3′ (nucleotides 1881–1858), was located between nucleotides 1357 and 1881 of the rat sequence. The PCR amplification was run at 94°, 54°, and 72° for 30 cycles (1-min each), as described (21).

Cloning and sequencing. Plaques from a human substantia nigra cDNA library (Clontech) were transferred onto duplicate nitrocellulose filters (BAS 85; Schleicher & Schuell) that had been prehybridized at 42° for 2 hr in 35% formamide, 1× Denhardt's (0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 0.1% ficoll), 20 mM Tris-HCl, pH 7.4, 4× SSC (60 mM, Na_3 citrate, 0.6 M NaCl, pH 7.0), 0.01% SDS, 20 μg/ml salmon sperm DNA, 20 μg/ml yeast tRNA. Hybridization was carried overnight at 42°, in the same solution containing 10% dextran sulfate, in the presence of 5×10^5 cpm/ml nicked translated probe A or B (specific activity, $2–4 \times 10^9$ cpm/μg of DNA). Filters were washed twice for 15 min in 2× SSC, 0.1% SDS, at 42° and then in 0.2× SSC, 0.1% SDS, at 42° (15 min) and 46° (15 min). Positive clones were plaque purified, and the corresponding *Eco*RI fragments were subcloned in pBluescript (Stratagene) for sequencing and preparation of probes (for the chromosomal assignment). A full length clone (clone 2–4) was sequenced in both orientations by the dideoxynucleotide chain termination method.

Chromosomal assignment and somatic cell hybrids. For these studies, two probes were used, a 3.5-kb clone comprising the full length coding region (clone 2–4), and a 1-kb clone corresponding to the beginning of the coding sequence (clone 1–4). For somatic cell hybridization (see Table 2), a mapping panel, consisting of 17 mouse-human (NA09925 to NA09938, NA09940, NA10324, and NA10567) and two Chinese hamster-human (NA10611 and GM07298) hybrids, was obtained from the National Institute of General Medical Sciences Mutant Cell Repository. Characterization of these hybrids and their human chromosome content are described in detail in the National Institute of General Medical Sciences Mutant Cell Repository catalog. For *in situ* hybridization, the 3.5-kb cDNA probe was nick-translated with [^{3}H]dATP and [^{3}H]dCTP, to a specific activity of 3.4×10^7 cpm/μg. Hybridization to human metaphase chromosome spreads, post-hybridization wash, emulsion autoradiography, and silver grain analysis were carried out as previously described (22).

Stable cell line transfection and expression. A full length *Eco*RI 3.5-kb fragment from clone 2–4 was excised from pBluescript and subcloned in the expression vector pCMV5 (17), to provide the pCMV5-HBT construct. The cDNA fragment comprises neither a poly(A)$^+$ tail nor a clear polyadenylation consensus signal but was inserted 5′ of the polyadenylation signal of the human growth hormone gene contained in pCMV5. Stable cell lines expressing the human or the rat DAT were obtained by transfection of mouse fibroblast (Ltk$^-$) cells with the pCMV5-HBT construct or pCMV5-TS3 construct, respectively. Both cell lines were simultaneously cotransfected with the selection plasmid pRSVNeo, by the calcium phosphate method (Bethesda Research Laboratories). The level of DA uptake was then assayed in the clonal cell lines that were resistant to 0.4 mg/ml geneticin (GIBCO) in the medium. Established cell lines stably taking up 0.78 and 1 nmol of [^{3}H]DA/min/10^5 cells for the rat and for the human DAT, respectively, were selected for pharmacological studies.

[^{3}H]DA uptake experiments. Cells were distributed in 24-well plates, at a density of ≈10^5 cells/well, and were grown for 48 hr, up to ≈2.5×10^5 cells/well. For uptake experiments, the Dulbecco's modified Eagle medium was replaced by a buffer containing 5 mM Tris base, 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, and 5 mM D-glucose, (final pH, 7.1) (uptake buffer). At the end of incubation, the cells were washed three times with 0.5 ml of uptake buffer, resuspended in 0.4 ml of 1% SDS, and left at 37°, with gentle shaking, for 1 hr. An aliquot was taken for scintillation counting. For uptake inhibition studies, cells were preincubated for 2 min in the presence of compounds to be tested, then 15 nM [^{3}H]DA was added in a final volume of 0.5 ml, and the incubation was continued for 4–5 min. In order to perform the most accurate comparison between the pharmacological values obtained for the rat and human DAT, experiments were done the same day with the same dilutions of drugs to be tested. To determine the Na^+ dependency, NaCl was substituted with 120 mM LiCl.

Results

Molecular cloning of a cDNA coding for the human DAT. A human substantia nigra library was screened with two probes derived from the rat DAT, which encompassed the first (TM-1) to the fourth (TM-4) TM domains (probe A) and the ninth TM domain (TM-9) up to the end of the coding sequence (probe B). Among 10^6 clones, three (1–4, 15–1, and 2–4) were found to hybridize with both probes A and B. Their *Eco*RI inserts, of 1, 1.2, and 3.5 kb, respectively, were subcloned into pBluescript for sequencing and analysis. The larger clone comprises an open reading frame of 1860 nucleotides (Fig. 1) starting with an ATG, which reasonably fits a Kozak consensus initiation site, and ending with a TAG stop codon at position 1861. The 3′ untranslated reading frame is not terminated by a poly(A)$^+$ tail, and a clear polyadenylation signal in this region is not apparent (data not shown). The putative protein encoded by this cDNA is 620 amino acids long, with a calculated molecular weight of 68,517. Hydropathicity analysis shows a pattern in which 12 21–24-hydrophobic amino acid segments could represent TM domains (Fig. 1). When compared with the

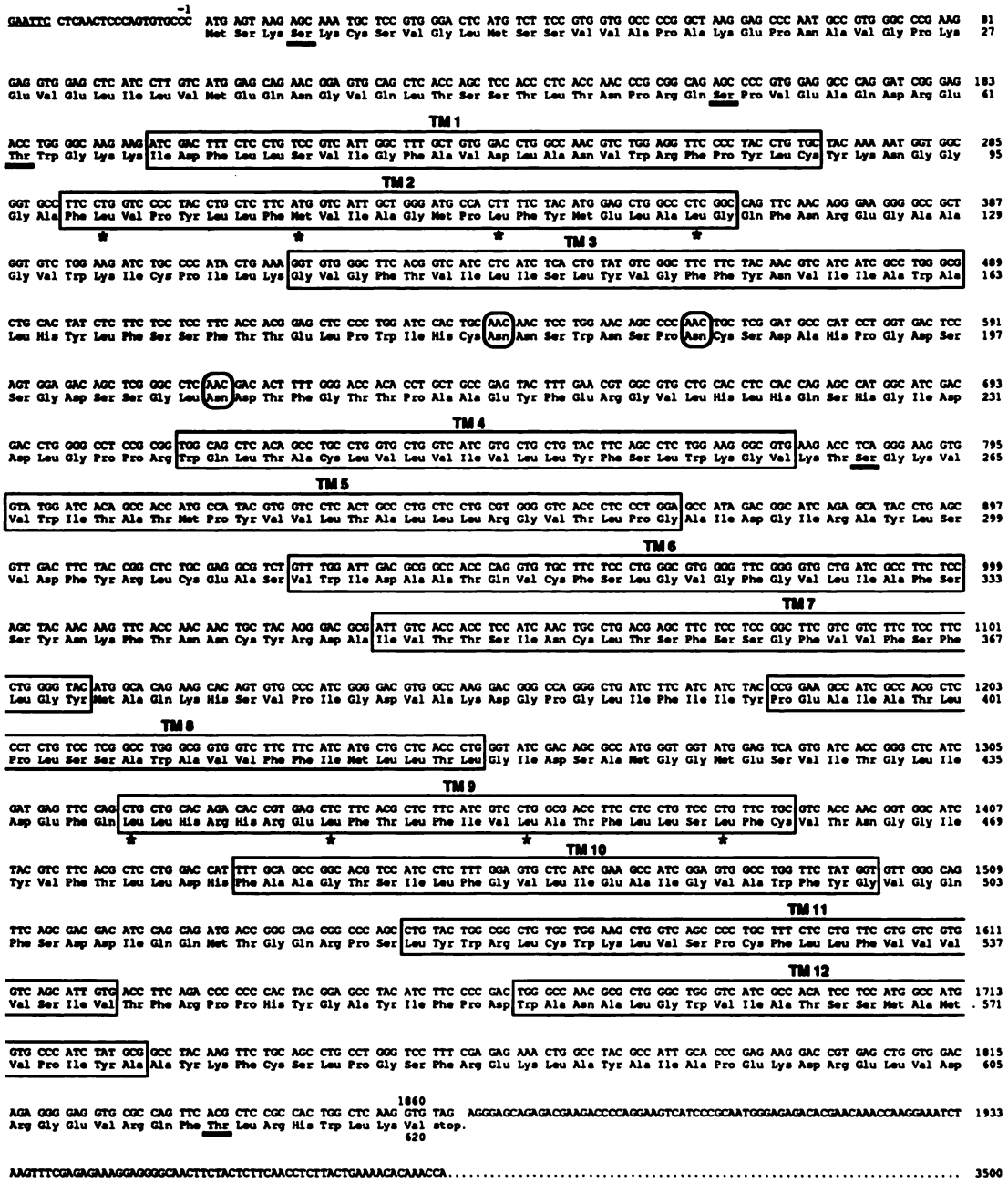

Fig. 1. Nucleic acid and deduced amino acid sequences of the human DAT cDNA clone. Nucleotides and amino acids are numbered (*right side*) consecutively from the putative start codon. The 12 TM domains are *boxed*. Serine and threonine residues existing in consensus phosphorylation motifs have been *underlined* (*solid bars* for PKA, *open bars* for PKC). *, Position of residues involved in the putative leucine/methionine zippers in TM-2 and -9. Potential asparagine glycosylation sites in the second extracellular loop are *circled*.

rat DAT, the human sequence possesses one extra glycine residue at position 199, in the third putative extracellular loop. The two transporters differ by 48 amino acid residues (Fig. 2), resulting in 92% overall identity. The bovine DAT has a 73-amino acid residue longer carboxyl-terminal tail than the human DAT, and an overall homology of 84%. Compared with other recently cloned neurotransmitter uptake sites, the greatest homology is found with the NAT (66% and 75% with conservative substitutions; see Fig. 2).

The human DAT possesses three potential glycosylation sites (asparagine residues 181, 188, and 205) in the large second extracellular loop, as compared with four in the rat DAT. According to consensus phosphorylation sites (23), three putative sites for PKA and two for PKC are found (Fig. 1). Interestingly, there are no potential glycosylation sites on the proposed intracytoplasmic loops and no putative phosphorylation sites on the extracytoplasmic loops. This observation is well in line with the 12-TM segment model, in which both the carboxyl-terminal and amino-terminal domains are located in the intracellular space. Also noticeable is the periodic repeat of leucine residues at every seventh position in the putative ninth TM segment. This type of structure has been referred to as a "leucine zipper" (24). Interestingly, in TM-2 a similar motif is observed; however, one of the leucine residues is substituted by a methionine.

Biochemical characterization of DA uptake. As shown

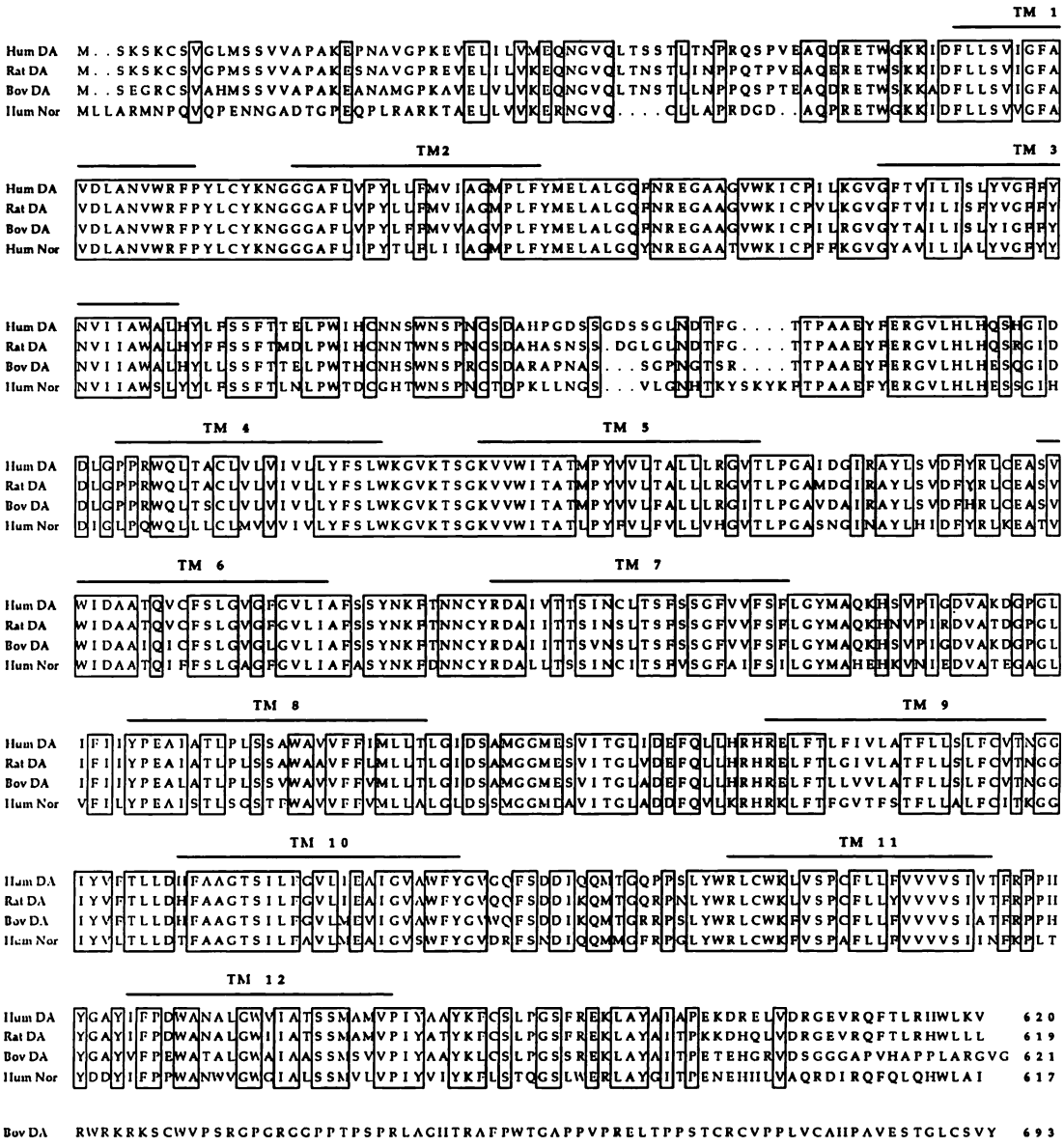

Fig. 2. Alignment of protein sequences from the human, bovine (20), and rat (17–19) DAT with the human norepinephrine transporter (14). The conserved regions are *boxed*. The putative transmembrane domains have been *overlined*. There is a possibility that the apparent longer carboxyl-terminal sequence for the bovine DAT might be due to a sequencing error, giving rise to a frame shift at residue 634 (T. Usdin and M. J. Brownstein, personal communication).

on Fig. 3A, transfection of Ltk$^-$ cells with the human DAT cDNA established in these cells a [^{3}H]DA uptake activity that was time and Na^+ dependent. Replacement of Na^+ by Li^+ in the uptake buffer dramatically decreased the uptake of [^{3}H]DA. At 37° and in the presence of 15 nM of [^{3}H]DA, the accumulation was linear over a period of 7–8 min and then a plateau was reached, lasting for at least 30 min. Because of these particular kinetic properties, the ability of various drugs to interfere with [^{3}H]DA transport was always determined after a 4–5-min incubation period. No significant DA uptake was observed in untransfected in Ltk$^-$ cells (data not shown).

Determination of the kinetic constants of uptake was performed by increasing the concentration of DA up to 30 μM (Fig. 3B). The Eadie-Hoftsee transformation of the data (Fig. 3C) yielded a monophasic curve, with an apparent dissociation constant, K_m, of 1.2 ± 0.3 μM and a maximal velocity, V_{max}, of 2.2 ± 0.5 nmol/min/10^5 cells, under the standard assay conditions (average ± standard error of five independent experiments, in triplicate).

Pharmacological characterization of DA uptake. Further characterization of human DAT was carried out by examining a large series of compounds for their ability to compete for [^{3}H]DA uptake. As shown in Fig. 4A, all compounds were able to compete with the labeled ligand, within about 2 orders of magnitude. This is consistent with the existence of a single site for these drugs on the transporter. The rank order of potency was mazindol = GBR12783 = GBR12909 = nomifensine > cocaine > bupropion > *d*-amphetamine > *l*-amphetamine, which matches closely the classical pharmacological profile for DA transport, as defined in the rat CNS. As shown in Fig. 4B, comparison of the K_i values (from Fig. 4A and Table 1) for the rat and the human DAT gave a correlation coefficient of 0.998 for >10 compounds.

Various "classical" tricyclic antidepressant molecules have a

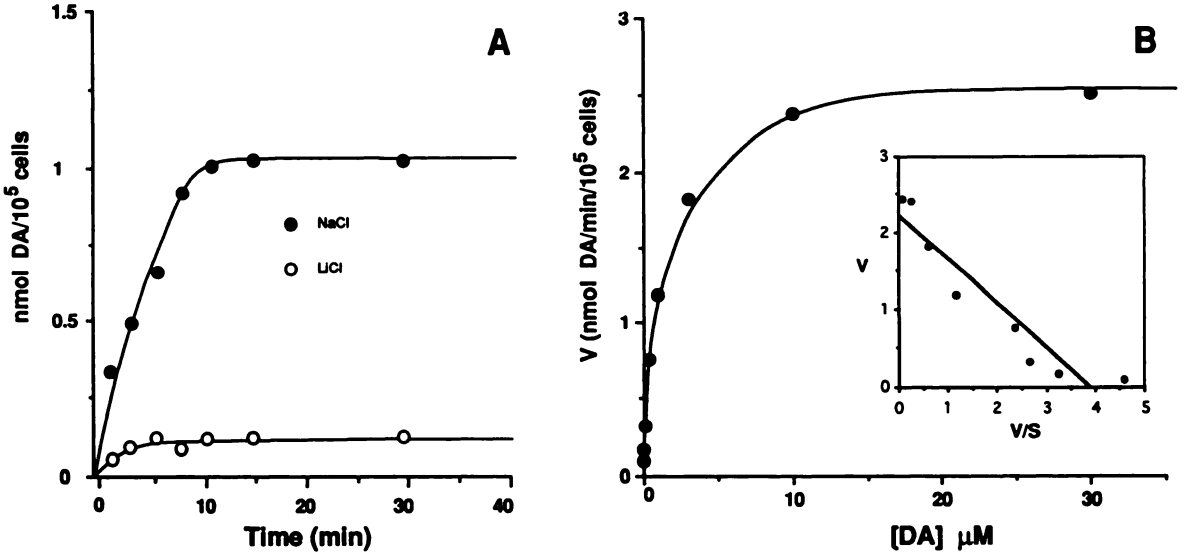

Fig. 3. Characterization of the human DAT permanently expressed in Ltk⁻ cells. A, Time course of [^{3}H]DA (15 nM) uptake in the presence of NaCl (●) or LiCl (○). B, Saturation of uptake by increasing concentrations of DA (0–30 μM). Results are the mean of three to five determinations, in triplicate.

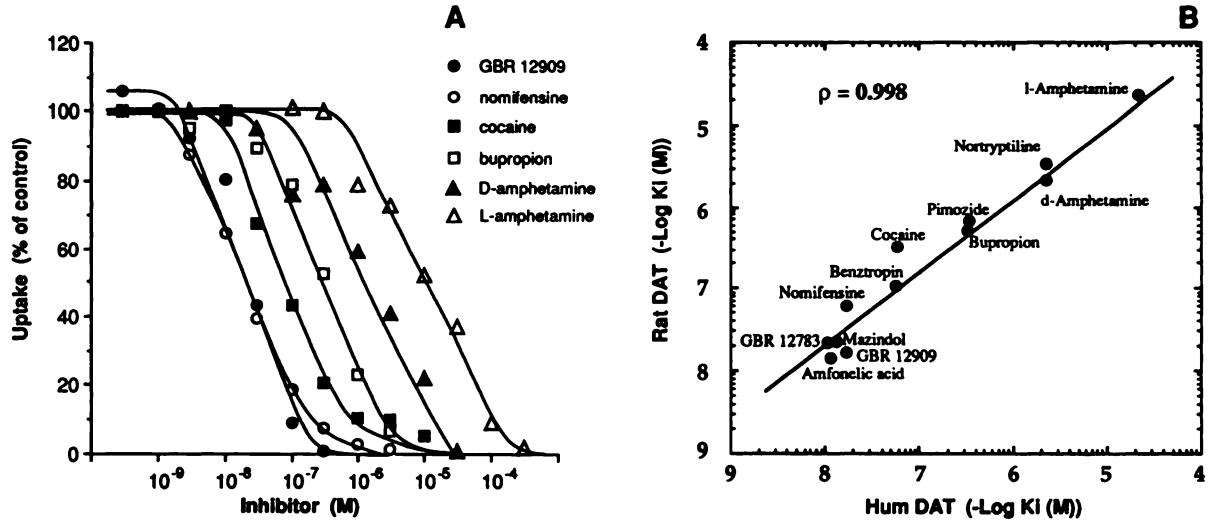

Fig. 4. Pharmacological characterization of the human DAT clone. A, [^{3}H]DA uptake competition assay in Ltk⁻ cells permanently transfected with the human DAT. The cells (≈2.5 × 10^5 cells/well) were preincubated with various concentrations of the compounds and then with [^{3}H]DA for 5 min, as described in Experimental Procedures. B, Correlation between the K_i values for different uptake inhibitors of the rat and human DAT expressed in Ltk⁻ cells. The K_i values were determined using the EBDA-LIGAND program (34). All curves were best fitted to a single class of inhibitor binding site. The results presented here are the means of three different experiments with seven concentrations of compound for each value.

moderate to low affinity for the human DAT. Moreover, the major anti-Parkinsonian drug L-DOPA is able to block DA reuptake, with a K_i above 10 μM. Finally, the efficacy of various neurotransmitters to displace DA uptake has also been determined on Ltk⁻ cells transfected with the human and rat DAT (Table 1); they all had a low affinity for the DAT.

Chromosome localization of the human DAT. *In situ* hybridization of the cDNA probe to normal human metaphase spreads revealed one specifically labeled site. Of 192 grains in 75 cells scored, 37 (19.3%) were found to be at the distal short arm of chromosome 5, band p15.2–15.3 (Fig. 5). No other chromosomal sites were labeled above background. Southern blot analysis of DNA from 19 rodent-human somatic cell hybrids confirmed the chromosome assignment made by *in situ* hybridization (Table 2). The 1-kb cDNA probe detected three *Eco*RI fragments of 3.6, 2.8, and 0.9 kb, six mouse-specific fragments of 2.8, 2.4, 1.9, 1.8, 1.5, and 0.9 kb, and five Chinese hamster fragments of 2.8, 2.1, 1.7, 1.55, and 0.9 kb (data not shown). Thus, only the 3.6-kb human fragment that was distinguishable in the hybrids was subject to discordance analysis. The 3.6-kb fragment was found to segregate with human chromosome 5 (Table 2). One hybrid, GM/NA09930A, reported to have human chromosome 5 present at a frequence of 0.12 failed to show specific hybridization, possibly due to the limited hybridization sensitivity.

Discussion

Because of its structural features, the human DAT can be included in the 12-TM domain sodium- and chloride-dependent transporter family. Although the first members of this group of proteins have been characterized only recently, the family

TABLE 1

Pharmacological characterization of human DAT and rat DAT permanently expressed in Ltk⁻ cells

Competition experiments were performed, as described for Fig. 4, in the presence of 15 nM [^{3}H]DA. All K_i values were determined using the EBDA-LIGAND program and represent the average of three to five independent experiments in triplicate determinations, with a standard error of <5% of the values reported.

Inhibitor	K_i of [^{3}H]DA uptake inhibition	
	Human DAT	Rat DAT
	nM	
Amfonelic acid	8.6	17.5
Mazindol	11.2	22.5
GBR 12783	12.5	22.5
GBR 12909	16.9	16.8
Nomifensine	17	60
Benztropin	55	109
Cocaine	58	322
Bupropion	330	521
Pimozide	344	667
PCP	430	677
d-Amphetamine	2,260	2,160
Nortriptyline	2,300	3,400
Amitryptiline	3,000	8,000
Zimelidine	6,000	25,000
Desipramine	13,000	12,000
l-Amphetamine	22,000	22,600
6-OH-DA	>10,000	>10,000
MPP^+	>10,000	>10,000
DA (K_m)	1,220	890
L-DOPA	>10,000	>10,000
GABA	>10,000	>10,000
NA	>10,000	>10,000
Serotonin	>10,000	>10,000
Adrenaline	>60,000	>400,000

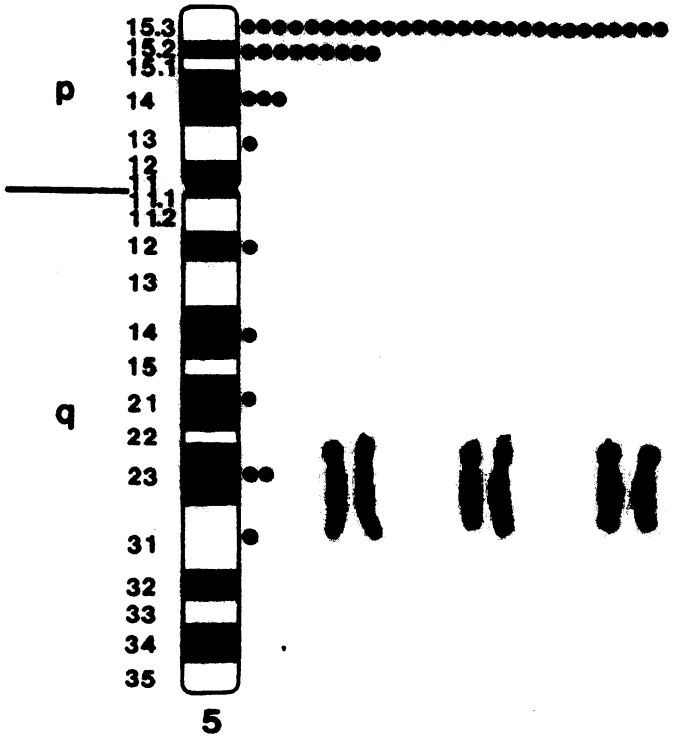

Fig. 5. *In situ* chromosomal assignment. Silver grain distribution along chromosome 5 after *in situ* hybridization with human DAT probes, illustrating the localization of a positive signal within the region 5p15.3. Three typical examples of positive labeling of chromosome 5 spreads are shown on the *right*.

already comprises more than eight members, counting species homologs, i. e., the rat and human GABA, the human norepinephrine, the rat serotonin, and, more recently, the rat and bovine DA and the rat proline transporters (12–20, 25). These proteins have several structural characteristics in common. The human DAT has three potential external glycosylation sites on the large putative extracellular second loop, a feature common to these transporters and classical for TM proteins. In addition, several consensus phosphorylation sites are found on the putative cytoplasmic domains of these proteins. Two PKA and one PKC consensus sites are found in the amino-terminus, in addition to one PKC site in the second intracytoplasmic loop and one site for PKA in the carboxyl-terminal tail. Most of these sites are also conserved in the rat DAT. These observations are somewhat unexpected, because DA transport has never been shown to be regulated by phosphorylation/dephosphorylation. Thus, an interesting prospect will be to study the activity of the human DAT in transfected cells after treatment with different protein kinase activators or inhibitors.

Another interesting structural feature is the presence of two leucine zipper motifs in TM-9 and TM-2. In the case of the TM-2 motif, the second leucine is replaced by a methionine. As pointed out by Landschulz *et al.* (24), methionine is the most suitable alternative residue to leucine. The leucine zipper in TM-2 is conserved among all cloned transporters except for the rat serotonin uptake site, whereas the one in TM-9 is found only in the human, rat, and bovine DAT. Leucine zippers are motifs that were originally identified and implicated in protein-protein interactions of certain DNA-binding proteins (24). However, it should be pointed out that the leucine zipper in TM-2 is interrupted by two prolines (amino acid residues, 101 and 112). The presence in the DAT proteins of one or two leucine zippers suggests that they might be used either for intramolecular bonding between TM-2 and -9 or for homologous or heterologous intermolecular polymerization. If such a role exists for these motifs, clusters of DAT might form on dopaminergic nerve endings, especially at the synapse. Such clustering of DAT molecules could theoretically greatly improve the efficiency of the uptake process at the synaptic level.

Most of the knowledge that has been accumulated about DA uptake has been obtained from studies on rat striatal synaptosomes. Because of the experimental difficulties in the preparation of reliable human synaptosomes, only a few attempts have been made to characterize DA transport in humans (26), and a complete pharmacological study has never been performed. More recently, such attempts were performed by injecting *Xenopus* oocytes with human substantia nigra mRNA (27). In this case, the difficulty of the assay did not allow extensive characterization. The molecular cloning of the human DAT allows, for the first time, direct assessment of the pharmacological characteristics of DA uptake in the human CNS. In order to have the most accurate determination of the pharmacological profile of human versus rat DA uptake, we established stable cell lines expressing both DAT under conditions as similar as possible, (i.e., same expression vector, pCMV5, and same eukaryotic cell line, Ltk⁻ cells). For the purpose of comparison, clonal cell lines expressing similar levels of the rat and human DAT were chosen. The values that were obtained for the rat DAT expressed in Ltk⁻ cells are all in good agreement with the values published in studies using rat striatal

TABLE 2

Segregation of human sequences detected by human DAT probe with human-rodent somatic hybrids

Human chromosome distribution between the various hybrid cell lines was analyzed by Southern blot, using a partial cDNA coding for the human DAT. A positive hybridization signal corresponds to a reactive *Eco*RI human DNA fragment distinguishable from any mouse or hamster DNA fragment. Informative hybrids are those in which the considered human chromosome is present at a frequency of 0.1 or more.

Presence of sequence/ presence of chromosome	Human chromosomes																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
Concordant																								
+/+	2	3	3	4	8	7	4	5	0	1	3	7	2	6	4	1	8	4	3	5	7	5	0	3
−/−	9	8	5	5	9	6	4	4	10	7	7	7	7	4	4	9	4	7	7	5	8	8	6	10
Discordant																								
+/−	6	5	4	3	0	1	3	2	8	3	4	1	4	1	3	7	0	4	3	2	1	2	8	4
−/+	2	3	6	5	1	5	6	5	1	3	2	3	3	7	5	1	7	4	3	6	2	2	1	0
Total discordant hybrids	8	8	10	8	1	6	9	7	9	6	6	4	7	8	8	8	7	8	6	8	3	4	9	4
Total informative hybrids	19	19	18	17	18	19	17	16	19	14	16	18	16	18	16	18	19	19	16	18	18	17	15	17
% Discordant	42	42	56	47	6	32	53	44	47	43	38	22	44	44	50	44	37	42	38	44	17	24	60	14

synaptosomes (Table 1), thus validating the present attempt to investigate the human DAT characteristics in these cells.

It has long been suspected that the human DAT is the preferred endogenous target responsible for cocaine reinforcement in humans (28). A major drawback to this hypothesis came from the fact that in rats the affinity of cocaine is higher for NA than DA uptake sites. In the present study we demonstrate that cocaine has a higher affinity for the human than for the rat DAT (50 versus 320 nM, respectively; Table 1). The K_i value of cocaine for the cloned human NAT is 320 nM (14); this makes the human DAT the better target for cocaine, thus providing stronger support for the "DA hypothesis" of cocaine effects in humans. However, these differences are not important enough to exclude a dual interaction of cocaine at both transporters.

It has been observed that amphetamine can elicit schizophrenic behavior in nonpsychotic individuals or enhance schizophrenic syndromes in psychotic patients (for review, see Ref. 29). This is associated with a marked stereoselectivity, with *d*-amphetamine being more potent than its *l*-stereoisomer. Despite some early controversies, the same stereoselectivity is observed in the inhibition of DA uptake in rodents, whereas the same potency is found for both enantiomers for the inhibition of NA uptake (29). From these observations, it was concluded that the "amphetamine psychosis" was a consequence of DA rather than NA uptake inhibition. We hereby confirm the stereoselectivity of *d*-amphetamine versus *l*-amphetamine in the blockade of the DA uptake in humans (Table 2), confirming that human amphetamine psychosis is more likely the result of an effect on DA uptake. We also show directly that PCP, which is thought to act primarily on σ opioid receptors, can block DA uptake via the human DAT (7).

Some recently developed antidepressant drugs, like mazindol, nomifensine, and bupropion, have comparable affinity for the human DAT and NAT (Ref. 14 and Table 1). On the other hand, it is confirmed here that tricyclic antidepressants, like nortriptyline and desipramine, possess a low affinity for the human DAT and, therefore, are 10–100 times more selective for the human NAT than the human DAT. Despite these differences, because high doses are used in the clinic to alleviate depression symptoms (10) it should be considered that the DAT might also be blocked by most of these drugs.

In humans, monkeys, and mice, the MPTP metabolite MPP^+ accumulates into dopaminergic terminals and then produces symptoms very similar to those of Parkinson's disease (8, 30). Using synaptosomes, Javitch *et al.* (8) have found that the EC_{50} for the ability of MPP^+ to inhibit DA transport in rat striatal synaptosomes is 170 nM. Using Ltk^- cells transfected with the cloned rat or human DAT, values of 10 μM have been obtained. This discrepancy is presumably not due to any trivial explanation, because results similar to those of Javitch *et al.* can be obtained under the conditions used here, when using rat striatal synaptosomes are used.[1] These findings suggest either that the cell lines transfected with DAT are missing an essential component for MPP^+ uptake or that another subtype of DAT, which has a better affinity for MPP^+, is present in the brain. Interestingly, a similar shift in affinity between the cloned DAT and the rat striatal synaptosome DA uptake model is observed not only with putatively transported substances like 6-OHDA, L-dopa, or NA, but also with DA itself, for which the K_m value in synaptosomes is 100 nM, compared with 1.2 μM (Table 1).

As mentioned above, an impairment of the DAT could possibly be involved in the etiology of some major psychiatric disorders or neurological diseases. As an initial approach to the search for a linkage or association of these diseases with potential polymorphism of the DAT gene, we have determined its chromosome localization. *In situ* chromosomal mapping reveals that the gene encoding the human DAT is localized on the most distal part of chromosome 5, at 5p15.3. Interestingly, much attention has been devoted to chromosome 5, because a possible linkage with familial schizophrenia has been seen. However, this linkage, which was discovered with probes recognizing the long arm of chromosome 5, has not been confirmed in different sets of families (31, 32). These studies illustrate the potential difficulties encountered in the search for a single candidate gene responsible for a likely multidimensional disorder such as schizophrenia. In this respect, the characterization of a human DAT cDNA will permit us to look for cosegregation of the different alleles of the human DAT gene within different sets of well established families with schizophrenic members. It already appears that the DAT gene is highly polymorphic, and restriction fragment length polymorphisms have been found for restriction enzymes like *Taq*I and

[1] C. Pifl, B. Giros, and M. G. Caron, manuscript in preparation.

*Msp*I.[2] Interestingly, despite the fact that few gene assignments are known on the short arm of chromosome 5, the most frequent chromosome deletion in humans occurs at 5p14–5p15 (33). This 5p monosomy underlies the "cri du chat" syndrome (33), a severe condition with malformations and mental retardation.

Thus, cloning, isolation, and characterization of a cDNA for the human DAT, as well as its expression into host cells, represent a unique and valuable model system for a better understanding of the biochemical and pharmacological characteristics of this process. Moreover, the cloning of the human DAT and the localization of its corresponding gene on chromosome 5 will open the way to further characterization of this potentially important genetic marker.

References

1. Sibley, D. R., and F. J. Monsma. Molecular biology of dopamine receptors. *Trends Pharmacol. Sci.* **13**:61–69 (1992).
2. Kebabian, J., and D. B. Calne. Multiple receptors for dopamine. *Nature (Lond.)* **277**:93–96 (1979).
3. Delay, J., P. Deniker, and J. M. Harl. Utilisation en thérapeutique psychiatrique d'une phénotiazine d'action centrale élective (4560 RP). *Ann. Med. Psychol. (Paris)* **110**:112–117 (1952).
4. Seeman, P., M. Chau-Wong, J. Tedesco, and K. Wong. Brain receptors for antipsychotic drugs and dopamine: direct binding assay. *Proc. Natl. Acad. Sci. USA* **72**:4376–4380 (1975).
5. Walters, J. R., D. A. Bergstrom, J. H. Carlson, T. H. Chase, and A. R. Braun. D1 dopamine receptor activation required for postsynaptic expression of D2 agonist effects. *Science Washington D. C.* **236**:719–722 (1987).
6. Axelrod, J. The metabolism, storage and release of catecholamines. *Recent Prog. Horm. Res.* **21**:597–619 (1965).
7. Horn, A. S. Dopamine uptake: a review of progress in the last decade. *Prog. Neurobiol.* **34**:387–400 (1990).
8. Javitch, J. A., R. J. D'Amato, S. M. Strittmatter, and S. Snyder. Parkinsonism-inducing neurotoxin, *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine: uptake of the metabolite *N*-methyl-4-phenylpyridine by dopamine neurons explains selective toxicity. *Proc. Natl. Acad. Sci. USA* **82**:2173–2177 (1985).
9. Ehringer, H., and O. Hornykiewicz. Verteilung von Noradrenalin und Dopamin (3-hydroxytyramin) im gehirn des Menschen und ihr Verhalten bei erkrankungen des Extrapyramidalen Systems. *Klin. Wochenschr.* **38**:1236–1239 (1960).
10. Willner, P., R. Muscat, M. Papp, and D. Sampson. Dopamine, depression and anti-depressant drugs, in *The Mesolimbic Dopamine System: From Motivation to Action* (P. Willner and J. Scheel-Kruger, eds.), John Wiley & Sons, Ltd., New York, 387–410 (1991).
11. Kanner, B. I. Bioenergetics of neurotransmitter transport. *Biochim. Biophys. Acta* **726**:293–316 (1983).
12. Guastella, J., N. Nelson, H. Nelson, L. Czyzyk, S. Keynan, M. C. Miedel, N. Davidson, H. A. Lester, and B. I. Kanner. Cloning and expression of a rat brain GABA transporter. *Science (Washington D. C.)* **249**:1303–1306 (1990).
13. Nelson, H., S. Mandiyan, and N. Nelson. Cloning of the human brain GABA transporter. *FEBS Lett.* **269**:181–184 (1990).
14. Pacholczyk, T., R. D. Blakely, and S. G. Amara. Expression cloning of an antidepressant-sensitive human noradrenaline transporter. *Nature (Lond.)* **350**:350–353 (1990).
15. Hoffman, B. J., E. Mezey, and B. J. Brownstein. Cloning of a serotonin transporter affected by antidepressants. *Science (Washington D. C.)* **254**:579–580 (1991).
16. Blakely, R. D., H. E. Berson, R. T. Fremeau, M. G. Caron, M. M. Peck, H. K. Prince, and C. C. Bradley. Cloning and expression of a functional serotonin transporter from rat brain. *Nature (Lond.)* **354**:66–70 (1991).
17. Giros, B., S. El Mestikawy, L. Bertrand, and M. G. Caron. Cloning and functional characterization of a cocaine-sensitive dopamine transporter. *FEBS Lett.* **295**:149–154 (1991).
18. Kilty, J. E., D. Lorang, and S. G. Amara. Cloning and expression of a cocaine-sensitive rat dopamine transporter. *Science (Washington D. C.)* **254**:578–579 (1991).
19. Shimada, S., S. Kitayama, C.-H. Lin, A. Patel, E. Nanthakumar, P. Gregor, M. Kuhar, and G. Uhl. Cloning and expression of a cocaine-sensitive dopamine transporter complementary DNA. *Science (Washington D. C.)* **254**:576–578 (1991).
20. Usdin, T. B., E. Mezey, C. Chen, J. Brownstein, and B. J. Hoffman. Cloning of a cocaine sensitive bovine dopamine transporter. *Proc. Natl. Acad. Sci. USA* **88**:11168–11171 (1991).
21. Giros, B., P. Solokoff, M. P. Martres, J. F. Riou, L. J. Emorine, and J. C. Schwartz. Alternative splicing directs the expression of two D2 dopamine receptor isoforms. *Nature (Lond.)* **342**:923–926 (1989).
22. Yang-Feng, T. L., G. Floyd-Smith, M. Nemer, J. Drouin, and U. Francke. The pronatriodilatin gene is located on the distal short arm of human chromosome 1 and on mouse chromosome 4. *Am. J. Hum. Genet.* **37**:1117–1128 (1985).
23. Kennelly, P. J., and E. G. Krebs. Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. *J. Biol. Chem.* **266**:15555–15558 (1991).
24. Landschulz, W. H., P. F. Johnson, and S. L. Mcknight. The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. *Science (Washington D. C.)* **240**:1759–1764 (1988).
25. Fremeau, R. T., Jr., M. G. Caron, and R. O. Blakely. Molecular cloning and expression of a high affinity proline transporter expressed in putative glutamatergic pathways of rat brain. *Neuron* **8**:1–12 (1992).
26. Haberland, N., and L. Hetey. Studies in *postmortem* dopamine uptake. I. Kinetic characterization of the synaptosomal dopamine uptake in rat and human brain after *postmortem* storage and cryopreservation: comparison with noradrenaline and serotonin uptake. *J. Neural Transm.* **68**:289–301 (1987).
27. Bannon, M. J., C. H. Xue, K. Shibata, L. J. Dragovic, and G. Kapatos. Expression of a human cocaine-sensitive dopamine transporter in *Xenopus laevis* oocytes. *J. Neurochem.* **54**:706–708 (1990).
28. Kuhar, M. J., M. C. Ritz, and J. W. Boja. The dopamine hypothesis of the reinforcing properties of cocaine. *Trends Neurosci.* **14**:299–302 (1991).
29. Kokkinidis, L., and H. Anisman. Amphetamine models of paranoid schizophrenia: an overview and elaboration of animal experimentation. *Psychol. Bull.* **88**:551–579 (1980).
30. Schultz, W. MPTP-induced parkinsonism in monkeys: mechanism of action selectivity and pathophysiology. *Gen. Pharmacol.* **19**:153–161 (1988).
31. Sherrington, R., J. Brynjolfsson, H. Petursson, M. Potter, K. Dudleston, B. Barraclough, J. Wasmuth, M. Dobbs, and H. Gurling. Localization of a susceptibility locus for schizophrenia on chromosome 5. *Nature (Lond.)* **336**:164–167 (1988).
32. Kennedy, J. L., L. A. Giuffra, H. W. Moises, L. L. Cavalli-Sforza, A. J. Pakstis, J. R. Kidd, C. M. Castiglione, B. Sjogren, L. Wetterberg, and K. K. Kidd. Evidence against linkage of schizophrenia to markers on chromosome 5 in a northern Swedish pedigree. *Nature (Lond.)* **336**:167–170 (1988).
33. Lejeune, J., J. Lafourcade, R. Berger, J. Vialatte, M. Boeswillwald, P. Seringe, and R. Turpin. Trois cas de délétion partielle du bras court d'un chromosome 5. *C. R. Hebd. Seances Acad. Sci.* **257**:3098–3102 (1963).
34. Munson, P. J., and D. Rodbard. LIGAND: a versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.* **107**:220–239 (1980).

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