vivo with the signal intensity obtained from a volume selected at the same coordinates relative to the coil in a rectangular phantom containing a solution of KCl (50 mM) and glycogen (150 mM). The phantom was raised 2.5 cm above the surface of the coil to simulate liver depth. Spectrometer sensitivity was normalized between phantom and in vivo measurements with the signal from the formate sphere as a calibration standard. To correct for the liver size being smaller than the coil field of view in the y dimension, we obtained an xy image of the pulse-sequence signal volume with a 9-cm ^{1}H surface coil on a phantom filled with water. The liver outlines determined from in vivo imaging and the glycogen phantom outline were superimposed on the pulse-sequence image, and we used the relative integrals to obtain a correction factor. The mean correction factor was 23%, with a range of 3 to 47%. We assessed reproducibility of the glycogen concentration measurement by obtaining 11 pairs of glycogen concentration measurements at 45-min intervals on four subjects. Each of the subjects had fasted between 6 and 12 hours. Subjects were removed from the magnet and probe between measurements. The coefficient of variation between the pairs of measurements was 7%.

5. Clinical imaging of all patients was performed on a 1.5-T magnet (General Electric, Milwaukee, WI). Multiecho axial scanning was performed [total echo time, 20 ms/80 ms (and repetition time, 2 s)]. The data were transferred to an independent work station (I.S.G. Technologies, Mississauga, Canada) and processed with the CAMRA S200 Program (I.S.G. Technologies), which provided three-dimensional reconstruction and volume calculations. Accuracy of the measurement was assessed with water-filled phantoms of known volume and determined to be ±5% with a coefficient of variation of ±1%.
6. Rates of total glucose production were determined at t = 22, 43, and 67 hours by a primed (25 μCi)–continuous (0.25 μCi/min) infusion of [6-^{3}H]glucose for 2.5 hours into a deep ante decubital vein. Blood samples were obtained every 10 min during the last 40 min of infusion from a retrodorsal vein in the hand, which was warmed to 70°C to arterialize the blood samples. Plasma [^{3}H]glucose specific activity was determined after isolation of glucose by ion-exchange chromatography as described [R. Kreisberg, A. Siegal, W. Owen, *J. Clin. Endocrinol. Metab.* **34**, 876 (1972)]. The rate (R_a) of total glucose production was calculated from the equation: R_a = infusion rate of tracer (disintegrations per minute per kilogram per minute)/[6-^{3}H]glucose specific activity (disintegrations per minute per milligram). The protocol was approved by the Yale University Human Investigation Committee and informed consent was obtained from each subject.
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Cloning and Expression of a Cocaine-Sensitive Dopamine Transporter Complementary DNA

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A rat dopamine (DA) transporter complementary DNA has been isolated with combined complementary DNA homology and expression approaches. The DA transporter is a 619–amino acid protein with 12 hydrophobic putative membrane-spanning domains and homology to the norepinephrine and γ-aminobutyric acid transporters. The expressed complementary DNA confers transport of [^{3}H]DA in *Xenopus* oocytes and in COS cells. Binding of the cocaine analog [^{3}H]CFT {[^{3}H]2β-carbomethoxy-3β-(4-fluorophenyl)tropane} to transfected COS cell membranes yields a pharmacological profile similar to that in striatal membranes.

DOPAMINE TRANSPORTERS TERMINATE dopaminergic neurotransmission by Na^+- and Cl^--dependent reaccumulation of DA into presynaptic neurons (*1*). Cocaine and related drugs bind to these transporters in a fashion that correlates well with their behavioral reinforcing and psychomotor stimulant properties; these transporters are thus the principal brain "cocaine receptors" related to drug abuse (*2*).

To find cDNAs that encode members of this neurotransmitter transporter family and that are expressed in brain regions rich in dopaminergic neurons, 500,000 plaques of a size-selected λ-Zap II rat ventral midbrain cDNA library were screened with a radiolabeled oligonucleotide complementary to conserved segments of the norepinephrine (NE) and γ-aminobutyric acid (GABA) transporters and products of polymerase chain reaction (PCR) amplification of brain cDNA with transporter-specific oligonucleotides (*3–5*). Messenger RNA transcribed from one of the 27 hybridization-positive clones, DAT1, conferred consistent cocaine-blockable accumulation of [^{3}H]DA in the *Xenopus* oocyte uptake assay that was more than ten times background levels (*6, 7*).

pDAT1 contains a 3.4-kb cDNA insert with a 1857-bp open reading frame. Assignment of the first ATG as the translation initiation site, on the basis of resemblances to consensus sequences for translational initiation (*8*), results in a protein of 619 amino acids with a nonglycosylated molecular weight of 69,000 (Fig. 1). Hydrophobicity analysis reveals 12 hydrophobic segments long enough to form transmembrane domains. The predicted DAT1 protein lacks an identifiable signal sequence. It displays four potential sites for N-linked glycosylation (Fig. 1). This sequence shows 67% amino acid identity and 81% similarity with the human NE transporter (*3*), 45% identity and 67% similarity with the rat GABA

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transporter (*4*), and no substantial homology with other protein database sequences. Residues forming putative transmembrane domains are especially well conserved.

Northern analysis of mRNA extracted from midbrain and from brainstem preparations that contain this region reveals a single band with a motility corresponding to 3.7 kb. This mRNA is not found in lung, cerebral cortex, or cerebellum. In situ hybridization reveals hybridization overlying neurons of the substantia nigra pars compacta and ventral tegmental area.

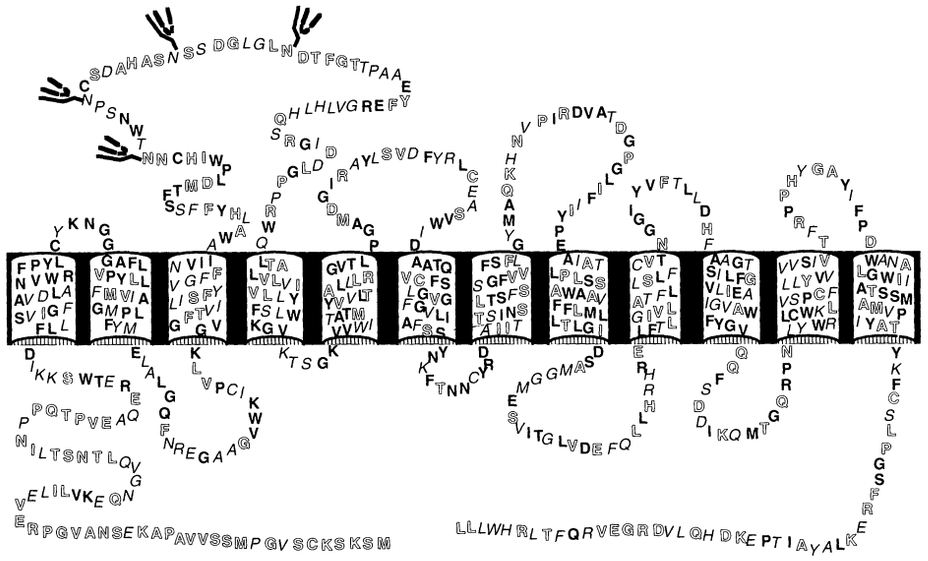

Fig. 1. Schematic representation of the dopamine transporter showing proposed orientation in the plasma membrane, amino acids conserved in GABA, DA, and NE transporters (dark letters), amino acids conserved in DA and NE transporters (italic letters), or amino acids found only in DAT1 (open letters) (*14*).

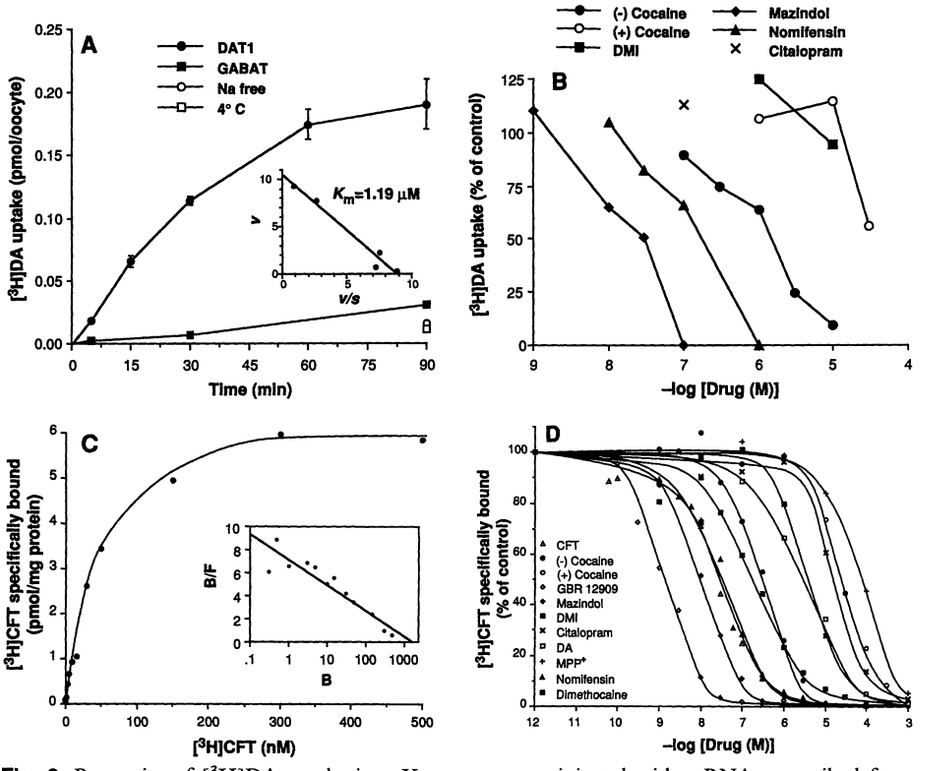

Fig. 2. Properties of [^{3}H]DA uptake into *Xenopus* oocytes injected with mRNA transcribed from pDAT1 and [^{3}H]CFT binding to COS cells transfected with pcDNADAT1. (**A**) Time-course and saturation analysis of [^{3}H]DA uptake into *Xenopus* oocytes injected with DAT1 mRNA or GABA transporter mRNA. (Inset) Eadie-Hofstee plot of initial velocity data at concentrations from 30 nM to 10 μM. (**B**) Pharmacologic profile of the abilities of various compounds to block [^{3}H]DA uptake into DAT1 mRNA-injected *Xenopus* oocytes at different concentrations. (**C**) Saturation analyses of [^{3}H]CFT binding to membranes of COS cells transfected with pcDNADAT1. (Inset) Scatchard plot of specific binding data. (**D**) Ligand competition for [^{3}H]CFT binding. Results shown are the means of triplicate determinations.

In *Xenopus* oocytes injected with mRNA transcribed from pDAT1, [^{3}H]DA is taken up with high affinity and Na^+, time, and temperature dependence (Fig. 2A) (*1, 3, 4*). Conversely, [^{3}H]DA is not accumulated in oocytes injected with an mRNA encoding the GABA transporter. The relative potencies of cocaine-like drugs in inhibiting [^{3}H]DA uptake in oocytes expressing DAT1 fit well with their relative potencies at the DA transporter in brain synaptosomes (Fig. 2B) (*1, 8*). The more active minus isomer of cocaine is almost two orders of magnitude more potent than the plus isomer. Mazindol displays a potency higher than that of cocaine, and agents primarily active at NE and serotonin (5HT) transporters, desmethylimipramine (DMI) and citalopram, show negligible potency (*1*). Oocytes injected with mRNA transcribed from pDAT1 do not accumulate [^{3}H]choline, glutamate, GABA, glycine, or adenosine. Inhibitors or competitors for transporters, hemicholinium-3, dihydrokainate, nipecotate, dipyridamole, glycine, and taurine fail to affect [^{3}H]DA accumulation into DAT1 expressing oocytes.

COS cells transfected with DAT1 cDNA subcloned into the eukaryotic expression vector pcDNA I (pcDNADAT1) display avid Na^+-, Cl^--, and temperature-dependent uptake of [^{3}H]DA. Transfected cells bind [^{3}H]CFT with a dissociation constant (K_D) of 46.5 ± 7.8 in tris buffers (*9*). These data fit a single site (Fig. 2), but binding in sucrose-phosphate buffers can reveal two sites (affinity estimates, 0.3 and 14 nM). Binding can be displaced by the cocaine stereoisomers GBR 12909, mazindol, and CFT with affinities that correlate well ($P < 0.0001$) with their affinities for striatal dopamine transporters (Fig. 2D) (*9, 11, 12*).

Thus, pDAT1 can be expressed to yield properties anticipated of brain DA transporter (*1*).

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CACCTCCGCCGTTCTTGTAGCACAGG-TAGGGGAAGCGCCACACGTT3′, which was based on sequences of NET, GABAT, and PCR products of library amplification derived with these sequences. Capped mRNA was synthesized from plasmids autoexcised from 27 positively hybridizing phage and tested for ability to confer uptake into injected oocytes. Messenger RNA from pDAT1 induced cocaine-blockable [^{3}H]DA uptake and was subjected to restriction mapping, automated sequencing of both strands, and confirmatory manual sequencing.

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Cloning and Expression of a Cocaine-Sensitive Rat Dopamine Transporter

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The action of dopamine and other monoamine neurotransmitters at synapses is terminated predominantly by high-affinity reuptake into presynaptic terminals by specific sodium-dependent neurotransmitter transport proteins. A complementary DNA encoding a rat dopamine transporter has been isolated that exhibits high sequence similarity with the previously cloned norepinephrine and γ-aminobutyric acid transporters. Transient expression of the complementary DNA in HeLa cells confirms the cocaine sensitivity of this transporter.

REUPTAKE SYSTEMS FOR THE BIOgenic amines have a central role in determining net synaptic activity and are the initial sites of action for a wide range of drugs with both therapeutic and abuse potential. Psychomotor stimulants such as cocaine and amphetamines act directly on neurotransmitter transporters to inhibit the reuptake of dopamine (DA), serotonin (5HT), and norepinephrine (NE). The reinforcing effects of cocaine, however, have been attributed to inhibition of DA reuptake in the nucleus accumbens and related targets of the mesolimbic DA system (*1*). The significance of the DA transporter to the addictive properties of cocaine provides impetus for understanding more about the structure, cellular physiology, and regulation of the transporter.

Degenerate oligonucleotides corresponding to regions of high sequence identity between the NE (*2*) and γ-amino-butyric acid (GABA) (*3*) transporters were used in polymerase chain reactions (PCR) with mRNA from midbrain (*4*). One PCR product, KW27, hybridized a 3.6-kb mRNA selectively expressed in the midbrain (*5*). A full-length clone (pDAT) was identified by high-stringency screening of midbrain and substantia nigra cDNA libraries with the KW27 probe (*6*). The deduced amino acid sequence (Fig. 1) reveals 12 hydrophobic segments and predicts the same topologic model as that suggested for the GABA and NE transporters with 12 transmembrane domains and both NH_2- and COOH-termini located cytoplasmically. The degree of sequence identity between pDAT and the NE and GABA transporters is 64 and 40%, respectively. One potentially significant difference is the presence of four predicted glycosylation sites in pDAT (Fig. 1), while the other two transporters have only three sites.

In situ hybridization studies provide further evidence that pDAT encodes a DA transporter. Brain regions known to contain dopaminergic neurons show specific hybridization to a KW27 cRNA probe and include the substantia nigra and ventral tegmental area, with less intense signals apparent in the periphery of the olfactory bulb and in discrete regions of the hypothalamus (*5, 7*). In order to confirm that pDAT represents the actual DA transporter and not a related gene product expressed in dopaminergic neurons, the transporter was expressed in HeLa cells with a T7-vaccinia virus transient expression system (*8*). Transfected HeLa cells demon-

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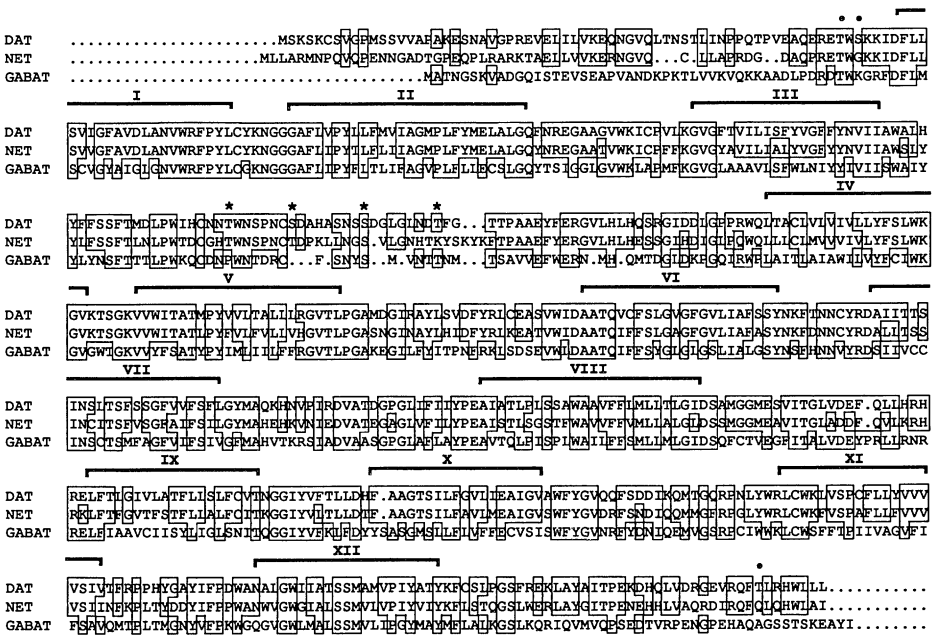

Fig. 1. Alignment of protein sequences of the rat DA transporter with the human NE (*2*) and GABA (*3*) transporters. Boxes, regions of identity; brackets, transmembrane helices; *, glycosylation sites on the putative extracellular loop. Two potential sites for phosphorylation by protein kinase C in the NH_2-terminal domain and one potential site for phosphorylation by either protein kinase C or Ca^{2+}-calmodulin–dependent kinase II in the COOH-terminal domain are indicated by solid dots.