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Short communication

Dopamine uptake and cocaine binding mechanisms: The involvement of charged amino acids from the transmembrane domains of the human dopamine transporter

Dalit E. Dar *, Thomas G. Metzger [1], David J. Vandenbergh [2], George R. Uhl

Molecular Neurobiology Branch, National Institute on Drug Abuse, Intramural Research Program, National Institutes of Health, Baltimore, MD 21224, USA

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Abstract

The wild type human dopamine transporter (DAT) and five DAT mutants were transfected into COS-7 cells and their ability to uptake dopamine or to bind cocaine was examine three days later. In each mutant, a single charged amino acid, located in areas that initial hydrophobic analysis had indicated were DAT transmembrane domains was substituted by alanine. Mutants used in this study were lysines 257 and 525 (termed K257A and K525A), arginines 283 and 521 (termed R283A and R521A), and glutamate 491 (termed E491A). Dopamine affinity was significantly enhanced in the K257A and R283A mutants, and the IC_{50} for displacement of the radioactive cocaine analog 2 beta-carbomethoxy-3 beta-(4-fluorophenyl)tropane (CFT) by cocaine was significantly elevated in the E491A mutant. All mutants displayed a reduction or complete loss of the maximal velocity (V_m) of dopamine transport.

Keywords: Dopamine transporter; Cocaine; Charged amino acid; Mutant; Glutamate

1. Introduction

The dopamine transporter (DAT) is the dopamine uptake site, and the binding site of cocaine (Ritz et al., 1987). Cocaine causes its euphoric and reinforcing effects by inhibiting the DAT-mediated reuptake of dopamine (Kuhar et al., 1991).

The DAT is a member of a family of Na^+ and Cl^- dependent biogenic amine transporters that also includes the norepinephrine transporter (NET) and the serotonin transporter (SERT) (Norregaard and Gether, 2001; Chen and Reith, 2000). Initial hydrophobicity analysis predicts these transporters to have 12 transmembrane domains with the N- and C-termini having an intracellular orientation (Pacholczyk et al., 1991). Recently, the bacterial leucine (Yamashita et al., 2005) transporter, $LeuT_{Aa}$, a homologue of the eukaryotic Na^+/Cl^- transporters, was crystallized and found to be composed of 12 transmembrane domains with intracellular N- and C-termini, as predicted. The $LeuT_{Aa}$ transmembrane domains discovered, however, only partially overlap with those suggested for the eukaryotic transporters.

Cloning of DAT cDNA (Shimada et al., 1991; Vandenbergh et al., 1992) has allowed more direct investigation of its structure, function and drug interactions. Since the crystal structure of the human DAT (hDAT) is not yet available, studies that mutate specific DAT amino acids are often used to elucidate DAT structure and function (Kitayama et al., 1992; Lin et al., 1999, 2000a,b). In previous mutagenesis studies, the roles of the DAT's aromatic (Lin et al., 1999, 2000b) cyclic (Lin et al., 2000a) and polar (Itokawa et al., 2000) residues were extensively investigated, but little information was gathered about the role of acidic (aspartate and glutamate) or basic (lysine, arginine and histidine) DAT amino acids. The exception was an

* Corresponding author. Current address: The Department of Neurobiology, The Weizmann Institute of Science, Arison Building, Room 204, Rehovot 76100, Israel. Tel.: +1 972 8 934 4418; fax: +1 972 8 934 4131.
E-mail address: dalit.dar@weizmann.ac.il (D.E. Dar).
[1] Current address: University of Southern Nevada, College of Pharmacy, II Sunset Way, Henderson, NV 89014, USA.
[2] Current address: Center for Developmental and Health Genetics and Department of Biobehavioral Health, The Pennsylvania State University, 101 Amy Gardener House, University Park, PA 16802, USA.

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early study examining DATs mutated at aspartic acid 79, which found this residue to be significant for both dopamine uptake and cocaine binding (Kitayama et al., 1992).

In this study, the role of charged DAT amino acids was investigated in relation to dopamine transport and cocaine binding mechanisms. According to all analyses, most DAT transmembrane domains consist primarily of aliphatic amino acids, and only a few charged amino acids. Charged DAT amino acids that are conserved in 2 or all of the biogenic amine transporters and that are located in the transmembrane domains, according to the initial hydrophobic analysis (Pacholczyk et al., 1991), were mutated to alanine, transfected to COS-7 cells and tested for their ability to bind cocaine or accumulate dopamine.

2. Experimental procedure

2.1. Materials

[^{3}H]CFT (84.5 ci/mmol) was from Perkin-Elmer Life Science (Boston, MA, USA). [^{3}H]dopamine (46 ci/mmol) was from Pharmacia Amersham (Amersham, England). Dulbecco's modified eagle's medium (DMEM), fetal calf serum (FCS) and trypsin/EDTA were from Life Technologies (Gaithesburg, MD, USA). Fish sperm DNA was from Hoffmann-La Roche Inc (Nutley, NJ, USA). Cocaine hydrochloride was supplied by the National Institute on Drug Abuse.

2.2. Preparation of DAT mutants

The cDNA that encodes wild type hDAT (Vandenbergh et al., 1992) in Bluescript vector (Stratagene, La Jolla, Ca, USA) was used for these mutagenesis studies. Prior to mutagenesis, the DAT insert was excised and cloned into pcDNA1 (Invitrogen, Carlsbad, Ca., USA) by digestion with NotI and xhoI, and ligated with T_4 DNA ligase (Invitrogen, Carlsbad, Ca., USA). A QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, Calif., USA) was used, with the template being DAT in a pcDNA1 background. Mutants expressed proteins with the same amino acid residues as did the DAT, except for a single amino acid that was replaced by alanine at the following locations: lysine at position 257, arginine at position 283, glutamate at position 491, arginine at position 521, or lysine at position 525. Oligonucleotides corresponding to the sequences for mutation were synthesized using an Applied Biosystems (Foster City, CA, USA) synthesizer and purified by electrophoresis using 12% polyacrylamide gels. The base sequences of the sense primers used (changed bases are underlined) were: 5′ GCTCTACTTCAGCCTCTGGGCGGGCGTGAAGACCTCAGGG 3′ for K257A, 5′ CCTCACTGCCCTGCTCCTGGCTGGGGTCACCCTCCCTGG 3′ for R283A, 5′ CCTCTTTGGAGTGCTCATCGCAGCCATCGGAGTGGCC 3′ for E491A, 5′ GCGGCCCAGCCTGTACTGGGCGCTGTGCTGGAAGCTGG 3′ for R521A and 5′ CTGGCGGCTGTGCTGGGCGCTGGTCAGCCCCTGC 3′ for K525A. Mutation-bearing fragments were cut using BglII and shuttled into the hDAT/pcDNA1 plasmid. MC1061/P3 supercompetent cells (Invitrogen transformation kit, Carlsbad, Ca., USA) were transformed with the wild type or mutant hDAT/pcDNA1, and plasmid preparations were carried out using a Qiagen plasmid kit (Qiagen, Valencia, CA, USA) to produce cDNA of purity suitable for transfection. The correct sequence and orientation of the full insert were confirmed by an ABI 377 automated sequencer (version 3.2, GMI, Ramsey, Minnesota, USA).

2.3. Cell transfection

COS-7 cells, cultured in DMEM supplemented with 10% FCS and 5% CO_2, were grown to confluence and then split in two. The next day, cells were harvested using trypsin/EDTA, centrifuged (200 ×g) for 10 min at 4 °C, and washed with cold, sterile HEBS buffer (in mM: NaHEPES 20, NaCl 130, KCl 5, Na_2HPO_4 0.7, glucose 6). The cells were resuspended in HEBS buffer at 2×10^7 cells/ml. Thereafter, 0.9 ml of suspension was transfected with 20 μg of pcDNA1 containing wild type or mutant DAT in the presence of 500 μg of fish sperm DNA using geneZAPPER 450/2500 (IBI, New Haven, CT, USA). Electroporation was carried out at 1100 μF and 300 V as described previously (Schaeffer et al., 1991). Transfected cells were suspended in DMEM and transferred to 12-well plates. After three days, cells were assayed for their pharmacological function. All measurements were performed using intact cells.

2.4. Cocaine binding and dopamine uptake

DAT-expressing cells were washed with Krebs–Ringer–Henselit (KRH) buffer (in mM: HEPES 25, NaCl 125, KCl 4.8, $CaCl_2$ 1.3, Mg_2SO_4 1.2, KH_2PO_4 1.2, glucose 5.6, pH = 7.4). They were then assayed to ascertain the ability of cocaine to inhibit the binding of 2 beta-carbomethoxy-3 beta-(4-fluorophenyl)tropane ([^{3}H]CFT) and to determine [^{3}H]dopamine uptake. For the binding assays, cells were incubated with 2 nM [^{3}H]CFT and unlabeled cocaine at concentrations ranging from 10^{-9} to 10^{-5} M for 2 h at 4 °C. For the uptake assays, cells were incubated with 20 nM [^{3}H]dopamine and 3×10^{-7} to 10^{-5} M non-labeled dopamine in 0.5 ml KRH buffer supplemented with 10 μM ascorbate for 5 min at 37 °C. Three washes with KRH terminated both binding and uptake assays. Cells were dissolved in 0.25 ml of 1% SDS, and their radioactivity was assessed. Mazindol (10^{-6} M) was added to parallel incubations to provide estimates of non-specific binding and uptake.

2.5. Data analysis

Graphpad Prizm Software (v2.01) was used to determine the values of K_m (Michaelis–Menten constant), V_m (maximum transport velocity) and IC_{50} (inhibitory concentration 50%). Statistical analyses, including one-way analysis of variance (ANOVA) with Tukey's post hoc tests, were applied using GraphPad Instat 3 for Windows 95.

3. Results

Lysine 257, arginine 283, glutamate 491, arginine 521 and lysine 525, which were presumed to be located within

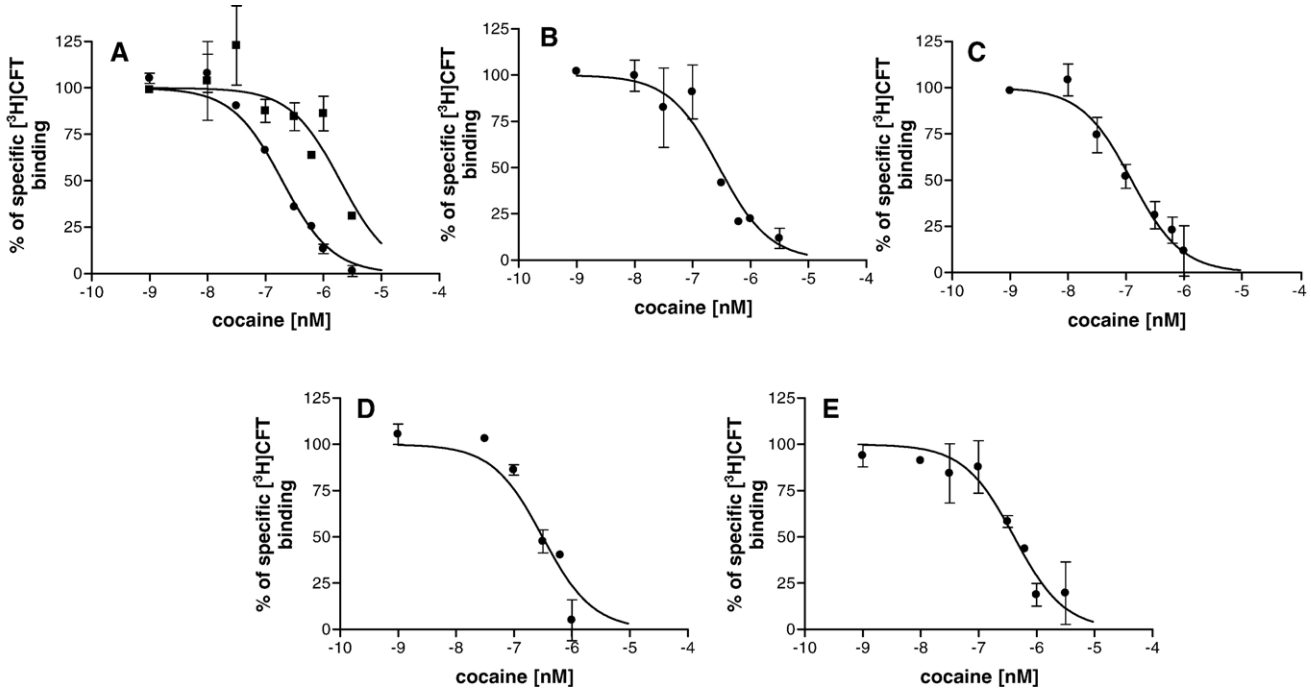

Fig. 1. Curves for cocaine displacement of radiolabeled CFT (2 nM) bound to COS-7 cells expressing the wild type DAT (●) or E491A (■) (A), K257A (B), R283A (C), R521A (D), K525A (E). IC_{50} values are presented in Table 1.

transmembrane (TM) domains 4, 5, 10 and 11, according to the initial hydrophobic analysis (Pacholczyk et al., 1991), were mutated to alanine. DAT mutants were expressed in COS-7 cells and tested for their ability to bind cocaine by displacing radiolabeled CFT, and for the ability to accumulate radiolabeled dopamine. Except from the E491A mutant, all DAT mutants displayed some degree of cocaine binding and dopamine uptake ability. DAT proteins were not immunostained in this study.

Mock pcDNA1 plasmid-transfected cells exhibited approximately 43% of the [^{3}H]CFT (2 nM) binding demonstrated by hDAT transfected cells ($n=1$, data not shown). Saturation curves using radioactive CFT were thus not produced. Instead, non-radiolabeled cocaine was used to provide estimates of the affinities for the mutated transporters. Mazindol was tested for its ability to displace [^{3}H]CFT bound to the mock transfected COS-7 cells (1 nM – 100 μM, $n=1$ data not shown). A concentration of 1 μM of mazindol, which did not displace [^{3}H]CFT in the mock transfected cells, was used to provide estimates of non-specific binding in the experiments presented. This concentration of mazindol does not take into account the low affinity site for ligands that was observed in a previous study (Dar et al., 2005).

The wild type DAT and all DAT mutants displayed cocaine inhibition curves that best fitted a one-site model (Fig. 1). The cocaine affinity displayed by the wild type human DAT (195.5±9 nM) is in the same range as that found in previous studies using a similar experimental setup (Pristupa et al., 1994). The affinity of cocaine for the E491A mutant was significantly (more than 10 fold) less than its affinity for the wild type DAT ($P<0.001$; Table 1, Fig. 1). The affinity of cocaine for the K257A, R521A and K525A mutants was reduced in comparison to its affinity for the wild type DAT, but not significantly so.

The wild type hDAT and all DAT mutants able to demonstrate dopamine translocation displayed saturation curves that best fitted a one-site model (Fig. 2). The K_m displayed by the wild type hDAT (3466±200 nM; Table 2) is in the same range as was found in previous studies using the same or other expressing cells (Pristupa et al., 1994; Giros et al., 1992). DAT mutants displayed a significantly lower dopamine translocation velocity, as estimated from their reduced V_m values in comparison to the wild type DAT ($P<0.001$; Table 2). The E491 mutant did not accumulate a measurable amount of dopamine, while the V_m values of the other mutants were 2.7 to 14 times lower than that displayed by the wild type DAT (Table 2, Fig. 2). The K_m of

Table 1
Effect of mutation of the dopamine transporter's charged amino acids on cocaine binding

Cocaine binding to:	hDAT	K257A	R283A	E491A	R521A	K525A
IC_{50} (nM) of cocaine	195.5±9	285±16	141.4±36	1962±372[a]	332±60	252.7±30

The ability of cocaine to inhibit the binding of 2 nM [^{3}H]CFT to the DAT in COS-7 cells transfected with pcDNA1 containing wild type or mutated DATs was determined. Inhibitory concentration 50% (IC_{50}) is presented as the mean± SEM from 3 or more experiments each performed in triplicate.
[a] $P<0.001$, Tukey test.

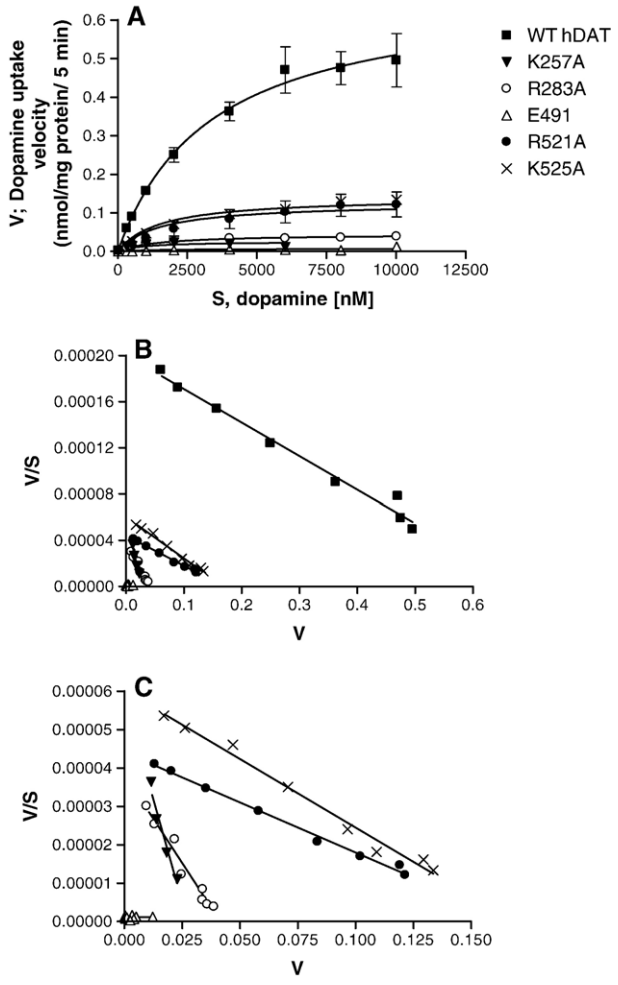

Fig. 2. (A) [^{3}H]dopamine uptake saturation curves performed on COS-7 cells expressing wild type DAT (■) or expressing DAT mutants K257A (▼), R283A (○), E491 (△), R521A (●) or K525A (x). Large (B)- and small (C)-scale Scatchard analyses from uptake experiments using wild type (B) and mutated DATs (B, C). K_m and V_m values are presented in Table 1.

dopamine for the K257A and R283A mutants was significantly lower (Fig. 2B,C) than for the wild type DAT ($P<0.001$; Table 2) as determined by the Scatshard transformation (Fig. 2).

4. Discussion

Hydrophobic analysis shows 2.7–5.4% of DAT transmembrane sequence to be comprised of charged amino acids (Pacholczyk et al., 1991; Goldberg et al., 2003), while $LeuT_{Aa}$ superposition puts the figure at 8.7% (Yamashita et al., 2005). According to the hydrophobic analysis conducted by Goldberg et al. (2003), all the described amino acids, except Lysine 257, are located within their related transmembrane domains. Lysine 257 is the first amino acid found outside the membrane, towards its inner part. When the recently elucidated bacterial $LeuT_{Aa}$ (Yamashita et al., 2005) is considered as a template, Lysine 257 is located in the short intracellular loop between TM4 and TM5, and arginine 283 is located in the extracellular loop between TM5 and TM6. The rest of the charged amino acids described are located in their related transmembrane domains, as was originally suggested (Pacholczyk et al., 1991).

Except for E491A, all tested mutants could bind to dopamine and cocaine, but the efficiency with which they could translocate dopamine through the membrane was significantly impaired. Mutants K257A and R283A displayed cocaine binding properties that are similar to those of the wild type DAT, yet they had decreased V_m and increased K_m values for dopamine transport. Previously, several studies suggested that there may be separate or overlapping, but not identical, cocaine and dopamine sites on the DAT (Kitayama et al., 1992; Lin et al., 1999, 2000a,b). Our results provide an additional indication that the cocaine binding site may be dissociated from the dopamine translocation site. The enhanced affinity of dopamine for the K257A and R283A mutants may indicate the existence of mutant-induced alterations in the secondary/tertiary structure of the DAT that affect the dopamine site, as has been suggested previously (Kitayama et al., 1993).

The affinity of cocaine for the E491A mutant was significantly reduced compared to its affinity for the wild type DAT. This mutant was also unable to transport dopamine. It is possible (as previously speculated for aspartic acid 79 (Kitayama et al., 1992)) that the carboxylic acids of aspartic acid 79 and glutamic acid 491 interact with dopamine's amine and with cocaine's nitrogen and so participate in dopamine transport and cocaine binding. From the structure of $LeuT_{Aa}$, TM10 is not expected to directly interact with the ligand or with sodium ions. However, it is possible that it is a part of the proposed extracellular gate (Yamashita et al., 2005).

Another possibility is that glutamic acid 491 can interact with one of the sodium ions. In a previous study, it was suggested that cocaine inhibits the uptake of dopamine by binding at the Na^+ binding site on the transporter (Coffey and Reith, 1994). If glutamic acid 491 has a role in binding Na^+ ions, this may explain its inability to translocate dopamine through the E491A mutant transporters (Table 2, Fig. 2). It may also underlie the

Table 2
Effect of mutation of the dopamine transporter's charged amino acids on dopamine uptake

Dopamine uptake by:	hDAT	K257A	R283A	E491A	R521A	K525A
K_m of dopamine (nM)	3466±200	506±61[a]	1201±94[a]	n.d.	3977±261	2947±127
V_m of dopamine (nmol/mg protein/5 min)	0.7334±0.049	0.039±0.008[a]	0.052±0.01[a]	n.d.	0.232±0.067[a]	0.273±0.055[a]

Cells were transfected with 20 μg of pcDNA1 containing wild type or mutated DATs and assayed for [^{3}H]dopamine uptake. Values for the Michaelis–Menten constant (K_m) and for the maximal velocity (V_m) of dopamine are presented as the mean±SEM from 3 or more independent experiments each performed in triplicate. n.d. = not determinable.

[a] $P<0.001$, Tukey test.

reduced cocaine binding affinities of this transporter (Table 1, Fig. 1). Finally, the disruption of normal DAT function by the E491A mutants may result from disruption of its 3-dimensional conformation or from the inability of this mutant to be expressed on the cell membrane.

5. Conclusions

Mutation of the DAT transmembrane domain charged amino acids targeted in this study resulted in a reduction or complete loss of the V_m of dopamine uptake. The affinity of dopamine for the K257A and R283A mutants was significantly enhanced, and the affinity of cocaine for the E491A mutant was significantly reduced, compared to their affinities for wild type DAT. The major findings of this study are that glutamate 491 is significant in the general functioning of the dopamine transporter and that the lysine and arginine mutants investigated do not affect the affinity of cocaine for the transporter, but do significantly impair the translocation of dopamine through the transporter.

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