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Dopamine Transporter Expression Confers Cytotoxicity to Low Doses of the Parkinsonism-inducing Neurotoxin 1-Methyl-4-phenylpyridinium

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The uptake of 1-methyl-4-phenylpyridinium (MPP^+), the active metabolite of the parkinsonism-inducing neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), was studied in various mammalian cell lines transfected, respectively, with the cloned human and rat dopamine transporters, and compared with rat striatal synaptosome preparations. Only in neuronally derived cell lines such as NG108-15, NS20Y, and SK-N-MC cells did MPP^+ have a K_M for the cloned transporters comparable to that of dopamine as seen in rat striatal synaptosomes. In non-neuronally derived cells such as COS-7, CHO, and Ltk$^-$ cells transiently or permanently expressing the transporters, the K_M of MPP^+ was at least 10-fold higher. The permanent expression of either the cloned human or rat dopamine transporters conferred to SK-N-MC cells susceptibility to the cytotoxic effects of low concentrations of MPP^+. The extent of this effect was dependent on the expression level of the dopamine transporters and could be specifically antagonized by the catecholamine uptake inhibitor mazindol. There were no significant differences in the susceptibility to MPP^+ of cells expressing similar levels of either the human or rat dopamine transporter. The demonstration for the first time of a quantitative relationship between the cellular expression of the plasma membrane transporter and the extent of the cytotoxic effects of MPP^+ suggests that known differences in vulnerability of various brain regions to MPP^+ cytotoxicity might be related to their actual content of dopamine uptake sites. In addition, our results suggest that intrinsic differences in the dopamine transporter proteins of humans and rats are probably not responsible for the marked increased susceptibility of primates to the neurotoxic effects of MPTP, as compared to rats.

[Key words: human dopamine transporter, rat dopamine transporter, MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), MPP^+ (1-methyl-4-phenylpyridinium ion), cytotoxicity, dopamine uptake, MPP^+ uptake, parkinsonism, transfection, cell culture]

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) produces parkinsonism in human and subhuman primates by a neurotoxic effect on dopaminergic neurons in the nigrostriatal pathway (Davis et al., 1979; Burns et al., 1983; Langston et al., 1983, 1984). MPTP is bioactivated in the brain by its oxidation to 1-methyl-4-phenylpyridinium ion (MPP^+), catalyzed by monoamine oxidase B (MAO-B) (Chiba et al., 1984; Heikkila et al., 1984b; Langston et al., 1984; Markey et al., 1984; Salach et al., 1984). A key event in the expression of the neurotoxicity of MPTP is the active uptake of MPP^+ into dopaminergic neurons as revealed by the protective effect of dopamine uptake blockers in MPTP-treated mice and monkeys (Javitch et al., 1985; Melamed et al., 1985; Schultz et al., 1986). Low doses of MPTP kill dopaminergic neurons in the substantia nigra of primates (Burns et al., 1983; Langston et al., 1984), much higher doses are required in mice (Heikkila et al., 1984a; Wallacc et al., 1984), and the rat is nearly insensitive to peripherally administered MPTP (Boyce et al., 1984; Sahgal et al., 1984). Several hypotheses about the differences in susceptibility of these species have been emphasized: differences in MPTP-binding sites, that is, MAO-B, in the substantia nigra (Javitch et al., 1984b; O'Carroll et al., 1987), differences in MPP^+-binding neuromelanin (D'Amato et al., 1986), and differences in MPTP-metabolizing MAO-B activity in the capillary wall of brain blood vessels (Kalaria and Harik, 1987).

Another possibility that has not been examined is that differences in the intrinsic properties of the uptake systems of these species exist with respect to MPP^+ as an uptake substrate. Until recently, the only model system for dopamine transport has been striatal synaptosomes. Comparative studies of rat versus human dopamine transport have been hampered by the problems in studying synaptosomal uptake in human tissue, that is, postmortem tissue. The cloning of both rat (Giros et al., 1991; Kilty et al., 1991; Shimada et al., 1991) and human dopamine transporters (Giros et al., 1992) allows controlled comparison of both uptake systems by expressing the cDNA and assaying the expressed transporter in the same cellular system (Giros et al., 1992). Besides the protective influence of dopamine uptake blockers on MPP^+ toxicity, it has never been demonstrated that toxicity is correlated directly with the expression of the dopamine transporter protein. Therefore, in order to examine these issues, we studied the uptake of ^3H-dopamine and ^3H-MPP^+ in various types of cells expressing various levels of either the cloned rat or the human dopamine transporter and examined cytotoxic effects of MPP^+ on such cell lines. To validate our uptake experiments we also measured transport in the classical model system of rat striatal synaptosomes.

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Materials and Methods

Materials. Sprague–Dawley rats were from Charles River. Media, sera, and other tissue culture reagents were obtained from GIBCO–Bethesda Research Labs. Drugs were obtained from Research Biochemicals, Inc. Radiochemicals were from New England Nuclear.

Synaptosomal preparation. Fresh crude synaptosomes were prepared according to the method described by Javitch et al. (1985) with minor modifications. Male Sprague–Dawley rats (250–350 gm) were killed by decapitation and the brains were chilled for 5 min in ice-cold PBS. The striatum was dissected at 4°C from 1-mm-thick coronal slices on an ice-chilled glass plate and homogenized in 15 vol (gm tissue/ml) of ice-cold 0.3 M sucrose in a tapered glass tissue grinder with a Teflon pestle (clearance of the cylindrical section, 0.1–0.15 mm; Wheaton). The homogenate was diluted 1:3 in 0.3 M sucrose and centrifuged at 1000 × *g* for 10 min. The supernatant was centrifuged at 12,000 × *g* for 20 min. The second pellet was resuspended in 30 vol of 0.3 M sucrose and used for uptake experiments.

Cell culture. COS-7 (African green monkey kidney), Ltk⁻ (mouse fibroblast), CHO (Chinese hamster ovary), NG 108-5 (mouse neuroblastoma × rat glioma hybrid), and NS20Y (mouse neuroblastoma) cells were grown in Dulbecco's modified Eagle's medium with L-glutamine and 4500 mg/liter D-glucose; the medium of COS-7 and Ltk⁻ cells also contained 10% heat-inactivated fetal bovine serum and 50 μg/ml gentamicin; that of NG108-15 cells, 5% heat-inactivated fetal bovine serum, 0.1 mM sodium hypoxanthine, 16 μM thymidine, and 1 μM aminopterin. Media for NS20Y cells contained 10% heat-inactivated fetal bovine serum and 110 mg/liter sodium pyruvate. SK-N-MC (human neuroblastoma) cells were grown in minimum essential medium with Earle's salts and L-glutamine, 10% heat-inactivated fetal bovine serum, and 50 μg/ml gentamicin. All cells were grown in 100- or 150-mm-diameter tissue culture dishes (polystyrene, Falcon) at 37°C under an atmosphere of 5% CO_2, 95% air, or in case of NS20Y cells under an atmosphere of 10% CO_2, 90% air.

Cell line transfection. The rat and human dopamine transporter cDNAs were used in the expression vector pCMV5 (Giros et al., 1991, 1992) for establishing the stable Ltk⁻ cell lines, whereas the human transporter cDNA was subcloned into pRc/CMV (Invitrogen Corporation) for transfection of all other cell lines. In order to obtain the human dopamine transporter in pRc/CMV, the cDNA subcloned in pBluescript (Giros et al., 1992) was amplified by polymerase chain reaction (PCR) as described (Giros et al., 1989) with two primers flanking the coding region. The primer in 5′ contained an Hind III site (GTAAAGCTTC AACTCCCAGTGTGCCCATG) whereas the primer in 3′ contained an Xba I site (GCGTCTAGACTTCCTGGGGTCTTCGTCTCTG). The amplified DNA was excised with the appropriate restriction enzymes and directionally subcloned into the corresponding sites of pRc/CMV. One clone was amplified and sequenced to check that there were no PCR errors. This clone was used for all the following transfections. For transient expression DEAE-dextran transfection was used for COS-7 and NS20Y cells, and calcium phosphate transfection was used for SK-N-MC and NG108-15 cells; 3 × 10^6 cells were inoculated in 100-mm-diameter dishes 1 d (COS-7, NG108-15 cells) or 2 d (NS20Y, SK-N-MC cells) before the transfection procedure. The DEAE-dextran transfection was started by washing the cells with PBS; then cells were incubated for 30 min with 5 ml of PBS containing 2.5 mg of DEAE-dextran and 15 μg of DNA at 37°C in the incubator. Twenty milliliters of medium containing 1.032 mg of chloroquine were then added and the cells incubated for 2.5 hr. After removing the medium, cells were treated for 2.5 min with 5 ml of medium containing 15% dimethyl sulfoxide, and incubated in medium overnight. The next morning cells from two 100-mm-diameter plates were distributed into three 24-well plates for uptake studies, which were carried out 2 d later. The calcium phosphate transfection was done according to the supplier's instruction (CellPhect transfection kit, Pharmacia). The cells of two 100-mm-diameter plates were distributed into three 24-well plates on the day after transfection. For stable transfection the calcium phosphate transfection system from Bethesda Research Labs was used; 1 × 10^6 cells were plated into 100-mm-diameter cell culture dishes 1 d (Ltk⁻, CHO) or 2 d (SK-N-MC) before transfection; 15 μg of human or rat dopamine transporter cDNA subcloned into pCMV5, 0.75 μg of pRSVNeo, and 5 μg of carrier DNA (salmon sperm DNA) were used per 100-mm-diameter dish of Ltk⁻ and CHO cells. SK-N-MC cells were transfected either with 15 μg of rat dopamine transporter cDNA subcloned into pCMV5, 0.75 μg of pRSVNeo, and 5 μg of carrier DNA, or with 15 μg of human dopamine transporter subcloned into pRc/CMV and 5 μg of carrier DNA. The day after transfection, cells were transferred into 150-mm-diameter dishes and were selected using 0.8 gm/liter G418 for Ltk⁻ and CHO cells and 1 gm/liter G418 for SK-N-MC cells. For uptake experiments cells were distributed into 24-well plates and the uptake was measured 2–3 d later.

Uptake into striatal synaptosomes. Twenty-five microliters of the synaptosomal suspension were added to 600 μl of uptake buffer (4 mM Tris-HCl, 6.25 mM HEPES, 120 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5.6 mM D-glucose, 0.5 mM ascorbic acid, final pH 7.1) containing 0.14 μCi of ^{3}H-dopamine (28 Ci/mmol) or 0.44 μCi of ^{3}H-MPP⁺ (84 Ci/mmol) and various concentrations of the drugs to be tested. After an incubation at 37°C for 5 min the uptake was stopped by the addition of 5 ml of ice-cold uptake buffer and immediate filtration through Whatman GF/B glass-fiber filters presoaked in 0.05% polyethylenimine. The filters were washed twice with 5 ml of ice-cold buffer and analyzed for tritium radioactivity in a Packard Tricarb 2000 CA liquid scintillation counter. Nonspecific uptake was estimated in the presence of 10 μM mazindol.

Uptake into cells. Uptake experiments were performed in 24-well plates. The uptake buffer was the same as was used for uptake into synaptosomes (see above). Each well was washed with 0.5 ml of buffer and incubated with 0.25 ml of buffer containing 0.7 μCi of ^{3}H-dopamine (28 Ci/mmol) or 2.2 μCi of ^{3}H-MPP⁺ (84 Ci/mmol) and various concentrations of drugs for 5 min. Uptake was stopped by aspirating the uptake buffer and washing each well twice with 1 ml of buffer. The radioactivity remaining in each well was determined by incubating with 0.4 ml of 1% SDS and transferring this solution into scintillation vials containing 10 ml of scintillation cocktail (Research Biochemicals Inc.).

Toxicity studies. Cells were distributed into 12-well plates (Ltk⁻ cells, 0.1 × 10^6 cells/well; SK-N-MC cells, 0.08 × 10^6 cells/well) and 3 d later different concentrations of MPP⁺ and/or mazindol or vehicle was added for various times. After the exposure, cells were recovered by detaching them with trypsin/EDTA and 1 ml of cell suspension was incubated with 2 μl of a saturated solution of the viability dye fluorescein diacetate in absolute ethanol (Rotman and Papermaster, 1966). The fluorescent cells were visualized under the fluorescence microscope and counted using a hemocytometer.

Data analysis. V_{max}, K_M, and K_i values were calculated by the iterative curve-fitting programs EBDA and LIGAND (McPherson, 1985).

Results

To compare uptake by the cloned transporters with the classical model of dopamine transport, synaptosomes from rat striatum were prepared. Dopamine and MPP⁺ were taken up by striatal synaptosomes with a K_M of 0.1 μM and 0.3 μM, respectively. The V_{max} of MPP⁺ uptake was about half of that of dopamine uptake (Fig. 1).

The rank order of potency of drugs inhibiting uptake of dopamine by the synaptosomal preparations (Table 1) was typical for a dopamine transporter: high affinity of the selective dopamine uptake blockers GBR12909 and benztropine and low affinity of potent blockers of 5-HT uptake—clomipramine—and noradrenaline uptake—desipramine. The K_i values of MPP⁺ for the dopamine transporter were in the same range as those of dopamine and noradrenaline.

The cloned rat and human dopamine transporters were expressed permanently in the same cell line, that is, Ltk⁻ cells. Uptake of dopamine and MPP⁺ was assayed under the same conditions as used for striatal synaptosomes. Transfected cell lines with comparable transport capacity for dopamine were studied and usually displayed an uptake of ^{3}H-dopamine or ^{3}H-MPP⁺ that was 10–20-fold higher than that observed in the presence of 10 μM mazindol or in untransfected cells. In isotopic dilution experiments the K_M of MPP⁺ for both rat and human dopamine transporters was much higher than that of dopamine (Fig. 2). In addition, the K_M of dopamine for the cloned transporters was significantly higher than that found in synaptosomal preparations. The V_{max} of MPP⁺ was not significantly different from that of dopamine, whereas the ratio of K_M values—K_M of

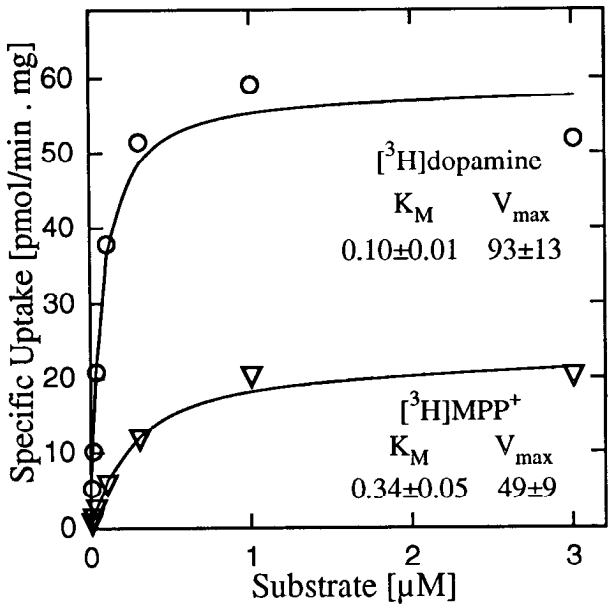

Figure 1. Uptake of ^{3}H-dopamine and ^{3}H-MPP$^+$ into rat striatal synaptosomes. The synaptosomal preparation (15–25 μg protein/tube) was incubated with 0.14 μCi of ^{3}H-dopamine or 0.44 μCi of ^{3}H-MPP$^+$ and various concentrations (0.03–10 μM) of dopamine or MPP$^+$, respectively, for 5 min at 37°C as described in Materials and Methods. Nonspecific uptake was measured in the presence of 10 μM mazindol. Shown is a representative saturation experiment, as well as the mean ± SE K_M (μM) and V_{max} (pmol/min·mg protein) of five independent experiments.

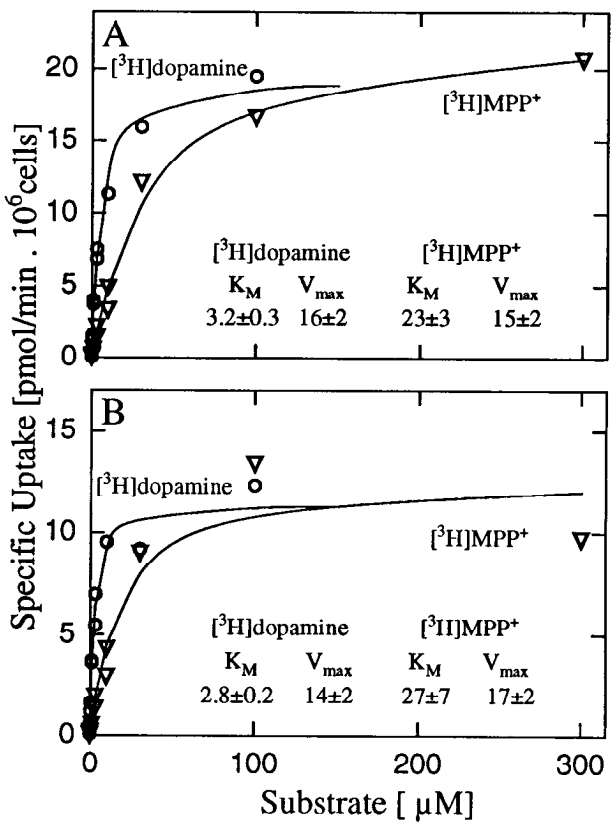

Figure 2. Uptake of ^{3}H-dopamine and ^{3}H-MPP$^+$ into Ltk$^-$ cells permanently transfected with the cloned human (*A*) or rat (*B*) dopamine transporter. The cells (0.15–0.25 × 10^6 cells/well) were incubated with 0.7 μCi of ^{3}H-dopamine or 2.2 μCi of ^{3}H-MPP$^+$ and various concentrations of dopamine (0.3–30 μM) or MPP$^+$ (1–300 μM), respectively, for 5 min at 37°C as described in Materials and Methods. Nonspecific uptake was measured in the presence of 10 μM mazindol. Shown is a representative saturation experiment, as well as the mean ± SE K_M (μM) and V_{max} (pmol/min·10^6 cells) of four independent experiments.

MPP$^+$ versus that of dopamine—was nearly 10. There was no significant species difference. In these cells the rank order of potency of uptake blockers was consistent with a specific dopamine transport system: high affinity of GBR12909 and benztropine, low affinity of clomipramine and desipramine (Table 1). Another substrate besides MPP$^+$ with high K_M for the cloned transporters was noradrenaline.

Ltk$^-$ cells expressing the cloned transporters were exposed to MPP$^+$ in the culture medium in concentrations up to 300 μM for up to 7 d. There was no difference in viability of these cells compared to untransfected Ltk$^-$ cells as tested by accumulation of fluorescein diacetate under the fluorescence microscope (data not shown). MPP$^+$ at 30 μM for 3 or 7 d, however, produced a change in pH of the culture medium of Ltk$^-$ cells expressing the cloned transporters toward acidic values as compared to vehicle treatment. There were no significant pH changes in untransfected Ltk$^-$ cells (Fig. 3). At high concentrations of MPP$^+$ (300 μM) an acidification of the medium of untransfected Ltk$^-$ cells was also observed. As a control, Ltk$^-$ cells transfected permanently with the D_{1A} dopamine receptor (Dearry et al., 1990) instead of the dopamine transporters using the same expression vector and selection principle, that is, pCMV5 and neomycin, reacted to MPP$^+$ exposure similarly as untransfected Ltk$^-$ cells (data not shown).

Table 1. Potencies of drugs inhibiting ^{3}H-dopamine uptake into rat striatal synaptosomes and into Ltk$^-$ cells expressing the cloned dopamine transporters

		Cloned transporter	
Inhibitor	Synaptosomes	Rat	Human
GBR 12909	4	13	17
Benztropine	63	110	56
Dopamine (K_M)	93	2800	3200
Noradrenaline	250	51,100	48,400
MPP$^+$	322	40,700	31,300
Clomipramine	2980	>1000	>1000
Desipramine	6330	13,000	12,000

^{3}H-dopamine uptake competition experiments were performed by incubation of synaptosomes with 5 nM and cells with 20 nM ^{3}H-dopamine in the presence of various concentrations of the compounds for 5 min at 37°C. Shown are mean k_i (K_M for dopamine) values (nM) of three independent experiments with a standard error of <7% of the values reported.

Obviously, *in vivo*, the dopamine transporter is expressed in neuronal cells, not in fibroblast like cells as Ltk$^-$. We therefore expressed the dopamine transporter in different cell types that, endogenously, were devoid of specific dopamine uptake (Table 2). In fact, whereas in all non-neuronal cell lines examined (transiently transfected COS-7 and permanently transfected Ltk$^-$ and CHO cells) MPP$^+$ showed a much higher K_i value than the K_M for dopamine (a ratio of about 10), in more neuronal-like cell types such as transiently transfected SK-N-MC, NS20Y, and NG108-15 this ratio of MPP$^+$ versus dopamine was similar to that in striatal synaptosomes. The K_i of noradrenaline was hardly shifted by the change of cellular expression system. Without transfection, all cell lines used showed an uptake of ^{3}H-dopa-

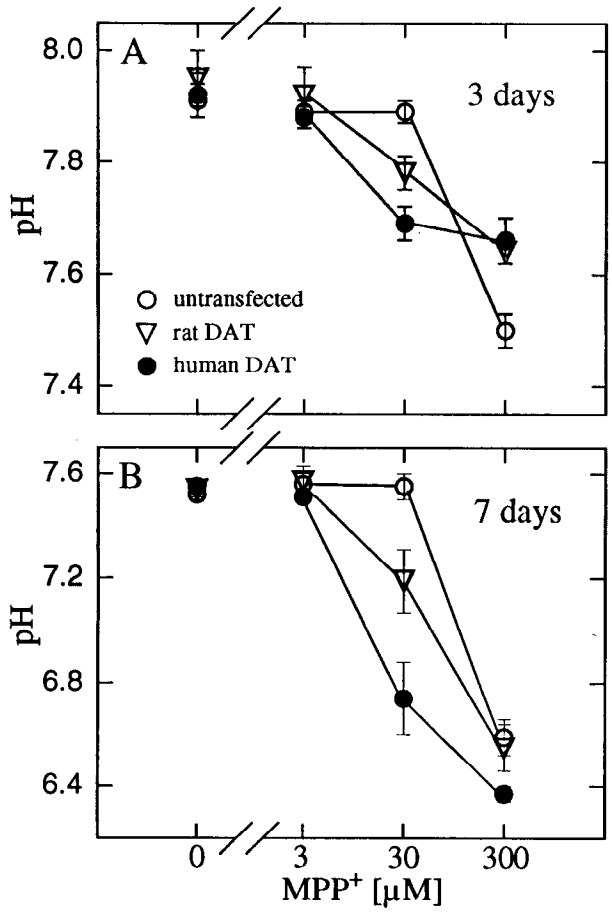

Figure 3. pH of the medium of Ltk⁻ cells expressing the rat and human dopamine transporters (*DAT*) after MPP⁺ treatment. Untransfected Ltk⁻ cells (*open circles*) and Ltk⁻ permanently expressing the cloned rat (*triangles*) and human (*solid circles*) dopamine transporter were distributed into 12-well plates (0.1×10^6 cells/well) and 3 d later various concentrations of MPP⁺ or vehicle were added to the medium. The pH of the medium of the cells was measured with a glass electrode after 3 d (*A*) or 7 d (*B*) of exposure to MPP⁺. Shown are mean values ± SE of three or four independent experiments.

mine that was similar to that of transfected cells in the presence of 10 μM mazindol. In addition, untransfected cells showed no difference in the nonspecific accumulation of MPP⁺ (data not shown).

To study the effect of MPP⁺ on neuronal cell lines expressing the dopamine transporters, we established SK-N-MC cell lines permanently expressing the dopamine transporters. Clones of different expression levels for rat and human dopamine transporters were obtained. Viability tests with fluorescein diacetate showed that 10 μM MPP⁺ in the medium for 3 d had no effect on untransfected SK-N-MC cells (Fig. 4*A,B*). SK-N-MC cells permanently expressing the human dopamine transporter showed no differences in morphology as compared to wild-type but there was a decreased number of fluorescent cells after a 3 d treatment with 10 μM MPP⁺ (Fig. 4*C,D*). The remaining fluorescent cells were of more elongated and spindly shape than the treated wild-type and untreated transfected cells. Cell counting in the hemocytometer after detaching the cells (Fig. 5) revealed toxic effects of MPP⁺ at concentrations of 1 μM for 3 d on cells expressing the highest level of uptake activity (50 or 82 pmol/min·10^6 cells for rat or human dopamine transporter, respectively). MPP⁺ in concentrations of 0.1–10 μM in the medium for 3 or 6 d had no

Table 2. Human dopamine transporter in different cell lines

	Dopamine K_M (μM)	MPP⁺ K_i (μM)	Noradrenaline K_i (μM)
COS-7	3.9 ± 0.7 (3)	23 ± 2 (3)	48 ± 5 (3)
Ltk⁻	2.9 ± 0.4 (5)	31 ± 6 (4)	48 ± 3 (3)
CHO	2.2 (1)	39 (1)	35 (1)
SK-N-MC	1.2 ± 0.2 (3)	3.7 ± 1.0 (3)	21, 19 (2)
NS 20 Y	1.6 ± 0.3 (3)	3.0 ± 1.1 (3)	19 ± 3 (3)
NG 108-15	3.1 ± 0.5 (3)	5.1 ± 1.5 (3)	31, 40 (2)

^{3}H-dopamine uptake competition experiments were performed by incubation of the transfected cells with 20 nM ^{3}H-dopamine in the presence of various concentrations of dopamine, MPP⁺, or noradrenaline for 5 min at 37°C. Shown are mean values ± SE of (*n*) independent experiments.

toxic effects on untransfected SK-N-MC cells. MPP⁺ at 100 μM had toxic effects after 6 d of exposure. Cells expressing a low uptake activity (0.9 pmol/min·10^6 cells) after transfection with the cDNA of the human dopamine transporter showed a decreased viability in 100 μM MPP⁺ after 3 d of exposure and in 10 μM MPP⁺ after 6 d exposure. Thus, there appears to be a direct correlation between the expression level of the dopamine transporter and the MPP⁺ cytotoxicity. The toxicity of 1 and 10 μM MPP⁺ in the highly expressing cells could be blocked by the catecholamine uptake inhibitor mazindol dose dependently (Fig. 6).

Discussion

Our study examined the properties of the cloned dopamine transporter as an influx pathway for MPP⁺, the toxic agent proper of the parkinsonism-inducing neurotoxin MPTP. The importance of the dopamine uptake system for the toxicity of MPTP has been suggested in several reports (Pileblad and Carlsson, 1985; Ricaurte et al., 1985; Sundström and Jonsson, 1985; Mayer et al., 1986) but never with respect to species differences and in systems where the total absence of the transporter could be tested. The expression of the cloned human and rat transporters in cell lines allowed us a controlled comparison of the protein of these species, avoiding the imponderables of postmortem artifacts using striatal synaptosomes. In this model system, there were no significant differences in the handling of MPP⁺ by the transporters of the two species. Therefore, our results suggest that differences in the susceptibility of these two species to toxic effects of MPTP cannot be due to differences in intrinsic properties of their respective dopamine transporter.

A puzzling finding, though, was the high K_M of MPP⁺ for the cloned transporters. Furthermore, the transporter did not confer to the Ltk⁻ cells a high sensitivity to MPP⁺. Ltk⁻ cells expressing the rat or the human dopamine transporter, exposed to MPP⁺ in concentrations up to 300 μM in the medium, did not die but showed a response consistent with a switch to anaerobic metabolism after shutting off the mitochondrial respiration by MPP⁺ (Vyas et al., 1986): an acidification of the medium at concentrations of MPP⁺ that did not affect untransfected Ltk⁻ cells. In agreement with our K_M or K_i data, a rather high concentration of MPP⁺, 30 μM, was necessary to observe this effect. Since the dopamine transporter *in vivo* is confined to neuronal cells, we expressed its cDNA in neuroblastoma-derived cell lines. In fact, there was a clear difference between non-neuronal COS-7, Ltk⁻, and CHO cells on the one hand and neuroblastoma-derived NG108-15, NS20Y, and SK-M-MC cells on the other in the handling of MPP⁺ by the expressed dopamine transporter: the

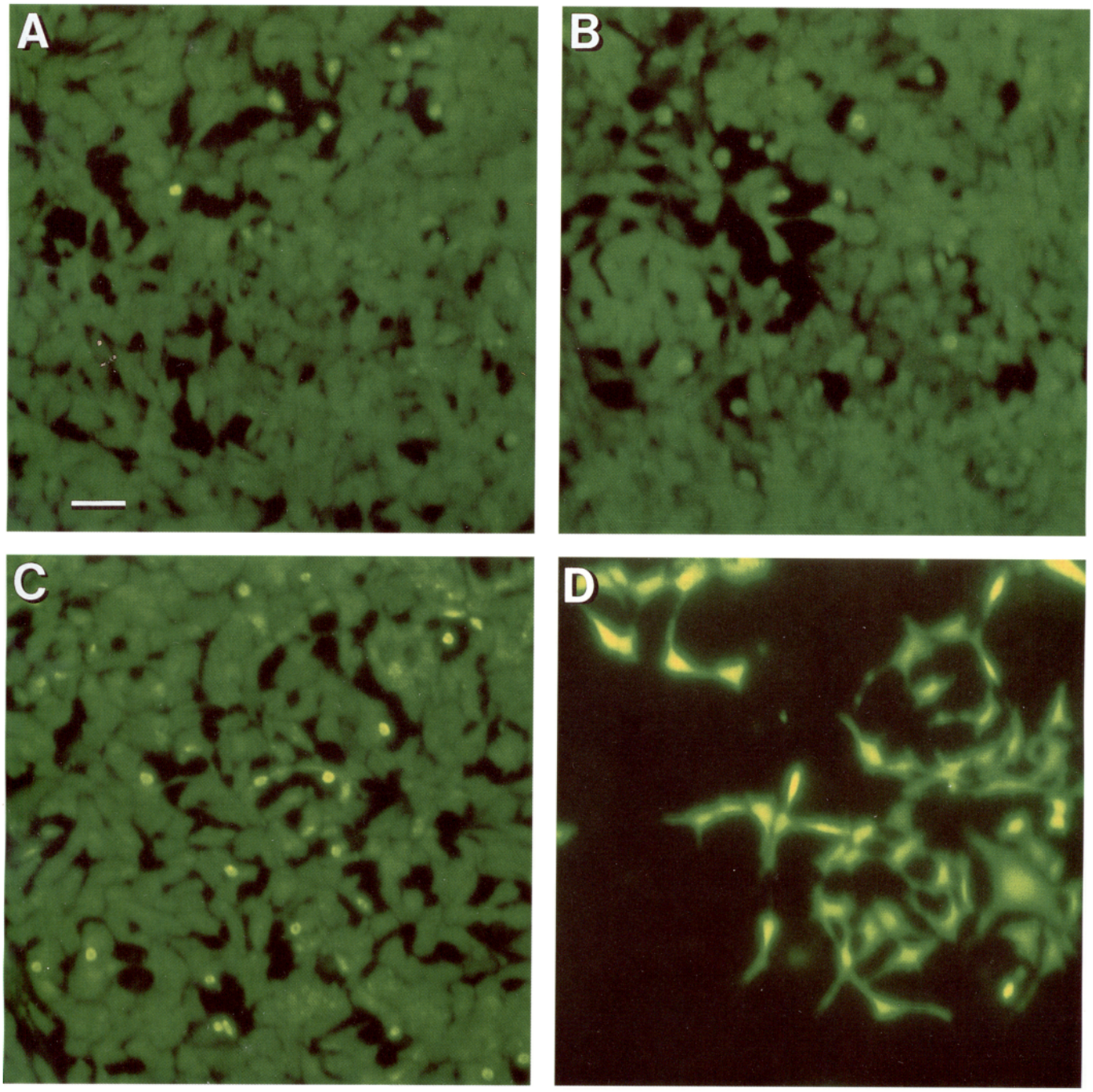

Figure 4. Effect of MPP+ exposure on the viability of SK-N-MC cells expressing the human dopamine transporter: fluorescence photomicrographs of untransfected SK-N-MC cells (*A, B*) and SK-N-MC cells transfected with the human dopamine transporter (*C, D*) stained with the viability dye fluorescein diacetate. Untransfected cells and cells expressing an uptake activity of 82 pmol/min·10^6 cells (V_{max}) were distributed in six-well plates (0.17×10^6 cells/well) and 3 d later vehicle (*A, C*) or 10 μM MPP+ (*B, D*) was added to the medium. After 4 d of exposure cells were incubated with fluorescein diacetate in fresh medium for 10 min, the medium was removed, and living cells were visualized under the fluorescence microscope. Scale bar, 20 μm.

K_i of MPP+ was lower in neuronal-like cells and similar to that of dopamine, resulting in a K_i ratio comparable to that in rat striatal synaptosomes. This change was the result of only the K_i of MPP+ being shifted considerably by the choice of expression system rather than that of the substrates dopamine or noradrenaline. This shift in K_i values can be due to a shift in the intrinsic affinity of MPP+ for the transporter. It is also possible that the cell membrane structure or posttranslational modifications in neuronal-like versus non-neuronal cells affect the transport of a lipophilic cation like MPP+ more than that of primary amines like dopamine or noradrenaline.

One general property of the cloned transporter expressed in cell lines is that the K_M of substrates (i.e., compounds actually taken up by the transporter such as dopamine, MPP+, and noradrenaline) are generally higher than in synaptosomal preparations. This property is also apparent if one examines previous

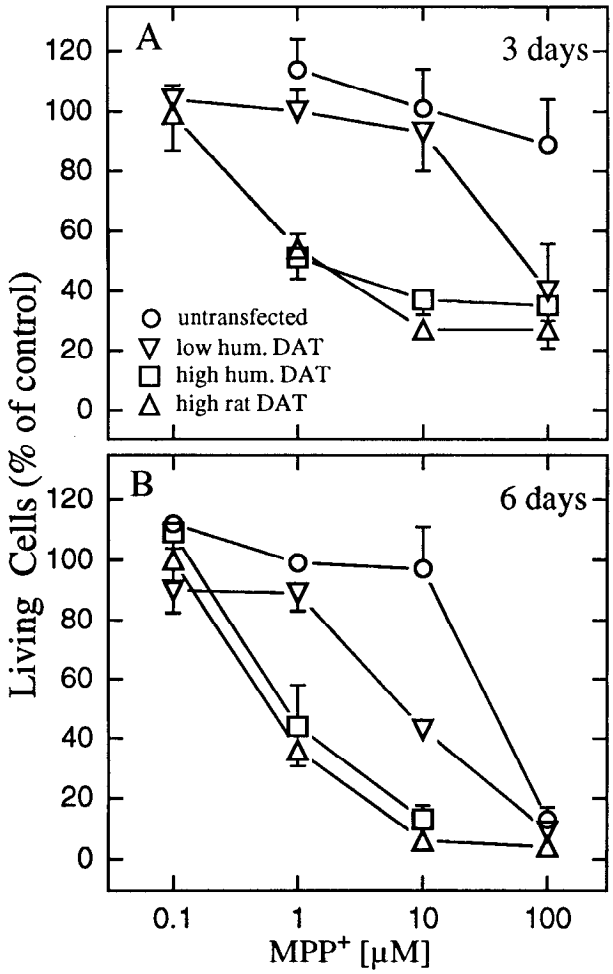

Figure 5. Toxicity of MPP^+ on SK-N-MC cells expressing the dopamine transporter (*DAT*). Untransfected SK-N-MC cells (*circles*) and SK-N-MC cells permanently expressing the cloned human (V_{max} of dopamine uptake: 0.9 pmol/min·10^6 cells, *inverted triangles*; 82 pmol/min·10^6 cells, *squares*), and rat (50 pmol/min·10^6 cells, *triangles*) dopamine transporter were distributed into 12-well plates (0.08 × 10^6 cells/well) and 3 d later various concentrations of MPP^+ or vehicle were added to the medium. After 3 d (*A*) or 6 d (*B*) of exposure, cells were recovered by detaching them with trypsin/EDTA, incubated with fluorescein diacetate, and fluorescent cells were counted with a hemocytometer under the fluorescence microscope. Shown are mean values ± SE of fluorescent cells expressed as percentage of vehicle-treated cells from three or four independent experiments.

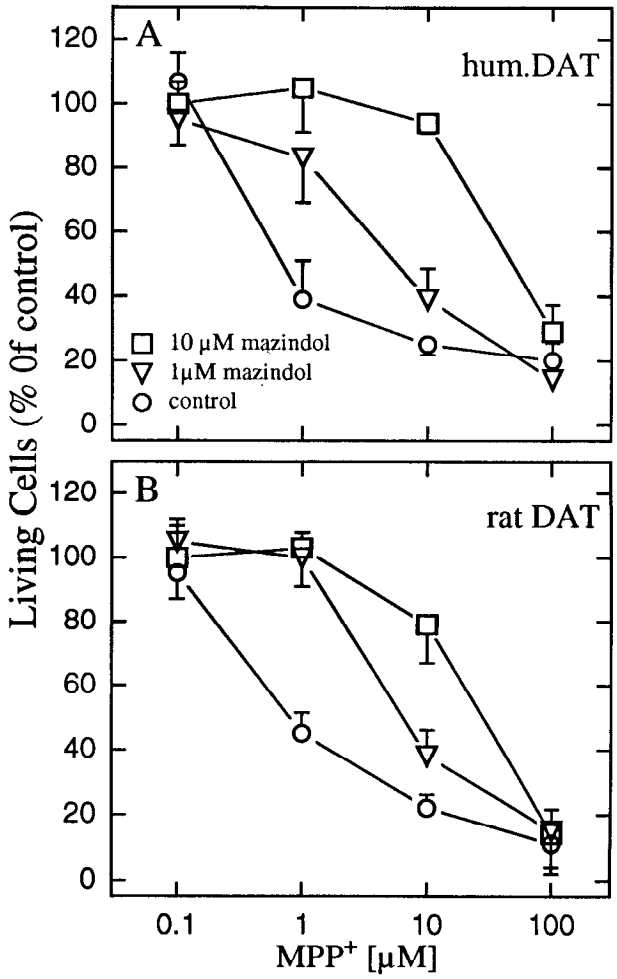

Figure 6. Blockade of the toxicity of MPP^+ by mazindol. SK-N-MC cells expressing the cloned human (*A*; 82 pmol/min·10^6 cells) and the rat (*B*; 50 pmol/min·10^6 cells) dopamine transporter (*DAT*) were distributed into 12-well plates (0.08 × 10^6 cells/well) and 3 d later, vehicle (*circles*) or 1 μM (*triangles*) or 10 μM (*squares*) mazindol and various concentrations of MPP^+ were added to the medium. After 4 d of exposure cells were recovered by detaching them with trypsin/EDTA, incubated with fluorescein diacetate, and fluorescent cells were counted with a hemocytometer under the fluorescence microscope. Shown are mean values ± SE of fluorescent cells expressed as percentage of vehicle-treated cells from three or four independent experiments.

reports for the rat dopamine transporter expressed in *Xenopus* oocytes (Shimada et al., 1991) and HeLa cells (Kilty et al., 1991) as well as for the noradrenaline transporter expressed in HeLa cells (Javitch et al., 1984a; Richelson and Pfenning, 1984; Pacholczyk et al., 1991). In our hands, we have also found the same relationship for the human noradrenaline transporter (gift of Dr. S. Amara, Vollum Institute, Portland, OR) expressed in COS-7 cells using the expression vector pRc/CMV as compared to rat synaptosomes (data not shown). A general shift in K_M of substrates for transporter proteins in transfected cells could be due to the different diffusion conditions of the substrate molecules, one-dimensional in case of transport into plated cells, three-dimensional in case of transport into spherical and much smaller synaptosomes. Alternatively, such differences might be due to potential differences (posttranslational modification, phosphorylation, protein–protein interactions) that might exist in the structural organization of the transporter at the synapse as compared to our cell models.

The dopamine transporter–expressing cells showed susceptibility to lower concentrations of MPP^+ than untransfected cells. Whereas in the case of Ltk^- cells the response to 30 μM MPP^+ consisted only of a transfection-specific acidification of the culture medium, there was clearly a decreased viability of transfected SK-N-MC cells in medium containing 1–10 μM MPP^+. There are several hypotheses for the initiation of cytotoxicity of MPP^+. One relates to the effective inhibition of mitochondrial respiration by MPP^+, which has been reported to be a selective complex I inhibitor in mitochondria (Nicklas et al., 1985; Poirier and Barbeau, 1985; Ramsay et al., 1986). The inhibition of the NADH ubiquinone oxidoreductase activity in complex I results in increased anaerobic glycolysis with lactate accumulation as shown in mouse neostriatal tissue slices incubated with MPP^+ (Vyas et al., 1986). This could explain the

acidification of the medium of Ltk⁻ cells incubated with MPP+ seen in the present study. The fact that Ltk⁻ cells did not die if incubated with up to 300 μM of MPP+ whereas SK-N-MC cells expressing even less dopamine transporter showed decreased viability in presence of 10 μM MPP+ points either to a greater ability of fibroblasts than that of neurons to form ATP anaerobically or to another hypothesis about mechanisms of MPP+ toxicity, that is, oxidative stress. Oxidative stress would be induced by intracellular redox cycling of MPP+, generating superoxide and toxic hydroxyl radicals (Sinha et al., 1986; Chacon et al., 1987). Our results of greater susceptibility of SK-N-MC cells than Ltk⁻ cells to toxic effects of MPP+ may be due to a different sensitivity to oxidative stress of these cell lines. It has been suggested that the nervous system is especially prone to radical damage. Its membrane lipids are very rich in polyunsaturated fatty acid side chains, which are especially sensitive to free radical attack (Halliwell, 1992), and the brain is poor in catalase activity and has only moderate amounts of superoxide dismutase and gluthatione peroxidase, enzymes that can protect against oxygen toxicity (Cohen, 1988). In fact, less basal gluthatione peroxidase activity was found in NS20Y neuroblastoma cells than in mouse L cells (Ceballos et al., 1988). The expression of the dopamine transporter in different cell lines provides a model to study the toxic mechanism of drugs that are taken up by this plasma membrane transporter inside the cell without recourse to high extracellular concentrations of these drugs, which could have nonspecific effects.

Previously, the association of MPP+ toxicity and uptake was observed in cultured neurons by the protective effects of uptake blockade (Sanchez-Ramos et al., 1986) and the relationship of toxicity and dopamine uptake affinity of structural analogs of MPP+ (Saporito et al., 1992). In PC12 cells, resistance to MPTP treatment leads to the selection of mutants lacking catecholamine uptake (Bitler et al., 1986). More recently, mazindol has been shown to block the MPP+-induced increase of lactic dehydrogenase release in COS cells transiently transfected with the rat dopamine transporter (Kitayama et al., 1992). Our observation that the toxicity of MPP+ on SK-N-MC cells expressing the rat or the human dopamine transporter could be prevented by the catecholamine uptake blocker mazindol reinforces these previous findings and suggests a cause/effect relationship between the transporter and MPP+.

Our study demonstrates for the first time that in cells permanently transfected with the dopamine transporter cDNA (i.e., a homogeneous population of cells), the sensitivity to toxic effects of MPP+ was dependent of the expression level of the dopamine transporter. Cells expressing a lower uptake activity withstood 1 μM of MPP+ for 6 d, a treatment that killed cells expressing higher uptake activity. Since there was also a clear uptake at 1 μM of MPP+ into the low-expressing cells, cells seem to be able to cope with a certain influx of the toxin. Only if this influx exceeds a certain threshold do toxic reactions become evident. Differences in the density of uptake sites per dopaminergic neuron could explain why various dopaminergic brain regions differ in their sensitivity to toxic effects of MPTP. For example, the nucleus accumbens shows a dopamine loss after MPTP (Mitchell et al., 1985) that in most studies is much smaller than that in the caudate or the putamen (Rose et al., 1989; Pifl et al., 1991). Several studies suggest a lower density of dopamine uptake sites in the nucleus accumbens or in the olfactory tubercle than in the caudate-putamen (Sershen et al., 1986; Marshall et al., 1990), quite apart from the fact that actual differences in transporter per nerve terminal could still be underestimated by binding studies of slices with labeled uptake blockers (Stamford et al., 1988; Wightman and Zimmerman, 1990).

In conclusion, the cloned dopamine transporters can confer, to neuronally derived cell lines that are permanently transfected, a susceptibility to the cytotoxic effects of *low* concentrations of MPP+. This provides a very flexible cellular model to study the cytotoxicity of these low doses of MPP+. There does not seem to be a decisive difference between the rat and the human dopamine transporter that could explain the differences in susceptibility of these species to irreversible damage by MPTP. Since our study demonstrated the relationship between the level of expression of transporters per cell and the manifestation of toxic effects of MPP+, it is possible that *in vivo* differences in the expression level of transporters per nerve terminal between different dopaminergic brain regions could explain their differences in susceptibility to MPTP exposure. Expression studies of the dopamine transporter in different cellular systems should give new insight into the vulnerability of different tissues to MPTP and, by consequence, insights into the mechanism of MPTP toxicity and possibly into the etiology of Parkinson disease.

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