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Dopamine transporter: involvement in selective dopaminergic neurotoxicity and degeneration

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Summary. The carrier molecule that transports dopamine (DA) into dopamine neurons by an electrogenic, Na^+- and Cl^--transport-coupled mechanism is known as the dopamine transporter (DAT). This uptake system is exclusively expressed in DA neurons with significantly higher levels of DAT expression in cells of the *substantia nigra pars compacta* than those of the ventral tegmental area and arcuate hypothalamic neurons. The expression density of DAT strongly correlates with the extent of DA cell loss in Parkinson's disease (PD). There are also DAT gene polymorphisms associated with PD. These data suggest a role of the DAT in the pathogenesis of PD. Though selective for its respective neurotransmitter, the DAT can also transport synthetic/natural analogues of the transmitter. Should such compounds interact with vital intracellular structures, their penetration into the neuron might have significant consequences. This sequence of toxic events could indeed demonstrated for the synthetic toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which produces selective degeneration of DA neurons characteristic of PD. Dopaminergic toxicity of its active metabolite 1-methyl-4-pyridinium (MPP^+) is mediated by the DAT through accumulation into DA neurons, where it inhibits mitochondrial complex I activity. Various endogenous and exogenous heterocyclic molecules, which are structurally related to MPTP/MPP^+, such as isoquinolines and β-carbolines, have been reported to exhibit similar toxic properties on DA cells, which are conferred by their uptake by the DAT. Taken together, there is large body of evidence from morphological, molecular biological and toxicological studies indicating that the DAT might be responsible for the selectivity of DA cell death in PD.

Keywords: Dopamine transporter, dopaminergic degeneration, neurotoxicity, Parkinson's disease.

Abbreviations

βC β-carboline, *DA* dopamine, *DAT* dopamine transporter, *HPP*$^+$ haloperidol pyridinium ion, *HEK-293* human embryonic kidney 293 cells, *HEK-hDAT* dopamine transporter transfected HEK-293 cells, *6-OHDA* 6-hydroxydopamine, *IQ* isoquinoline, *MPP*$^+$ 1-Methyl-4-phenylpyridinium ion, *MPTP* 1-Methyl-4-phenyl-2,3,6-tetrahydropyridine, *PD* Parkinson's disease, *SNpc* substantia nigra pars compacta, *TaClo* 1-trichloromethyl-1,2,3,4-tetrahydro-β-carboline, *THβC* 1,2,3,4-tetrahydro-β-carboline, *TIQ* 1,2,3,4-tetrahydroisoquinoline.

Introduction

The dopamine transporter (DAT) belongs to a large family of Na^+- and Cl^--dependent transporters including other monoamine transporters, such as the norepinephrine and serotonin transporter, as well as GABA and glycine carriers (Amara and Kuhar, 1993; Giros and Caron, 1993). The transport process of the DAT involves translocation of the substrate dopamine (DA) as well as two sodium and one chloride ions across the cell membrane (Krueger, 1990; McElvain and Schenk, 1992). The uptake is energetically coupled to transmembrane concentration gradient of Na^+, which is maintained by Na^+-K^+-ATPases (Hitri et al., 1994). The cDNAs encoding DAT have been cloned from various species including rat, mouse, and human tissue (Kilty et al., 1991; Usdin et al., 1991; Giros et al., 1992). The coding sequence for the DAT covers about 2 kb corresponding to 617 to 630 amino acid residues, resulting in molecular weights of the protein ranging from 60 to 97 kDa depending on glycosylation. Comparison of the primary sequences of monoamine transporters revealed between 69 and 80% homology between DAT and other monoamine transporters. Hydropathy analysis of the amino acid sequence of the DAT together with immunofluorescence and electron microscopy investigations suggest the presence of 12 transmembrane domains (TM) with the C- and N-terminus within the cytoplasm and a large extracellular loop between TM 3 and 4 (Fig. 1; (Amara and Kuhar, 1993; Giros and Caron, 1993; Nirenberg et al., 1996).

In mammalian brain, DAT mRNA is localized in cell bodies and is restricted to DA neurons (Amara and Kuhar, 1993; Augood et al., 1993; Lorang et al., 1994; Nirenberg et al., 1996). DAT mRNA has been detected in the *substantia nigra* (SN), the midbrain region and the brain stem with the highest expression levels in the *substantia nigra pars compacta* and *pars lateralis* and ventral tegmental area (VTA) (Usdin et al., 1991; Shimada et al., 1992; Cerruti et al., 1993b; Lorang et al., 1994). Co-localization with tyrosine hydroxylase immunostaining showed that the expression was localized only in cell bodies of DA neurons (Usdin et al., 1991; Augood et al., 1993; Hurd et al., 1994; Lorang et al., 1994). The first regional map of the distribution of the DAT protein in postmortem human brain using [^{3}H]mazindol showed DAT labeling most evident in the striatum and moderate labeling over DA cell bodies fields, including the SNpc, VTA and the retrorubral field (Donnan et al., 1991). Generation of antibodies highly specific to DAT allowed quantification and localization of the DAT protein (Nirenberg et al., 1996; Hersch et al., 1997; Ciliax et al., 1999). DAT immunoreactivity was concentrated in somatodendritic and terminal fields

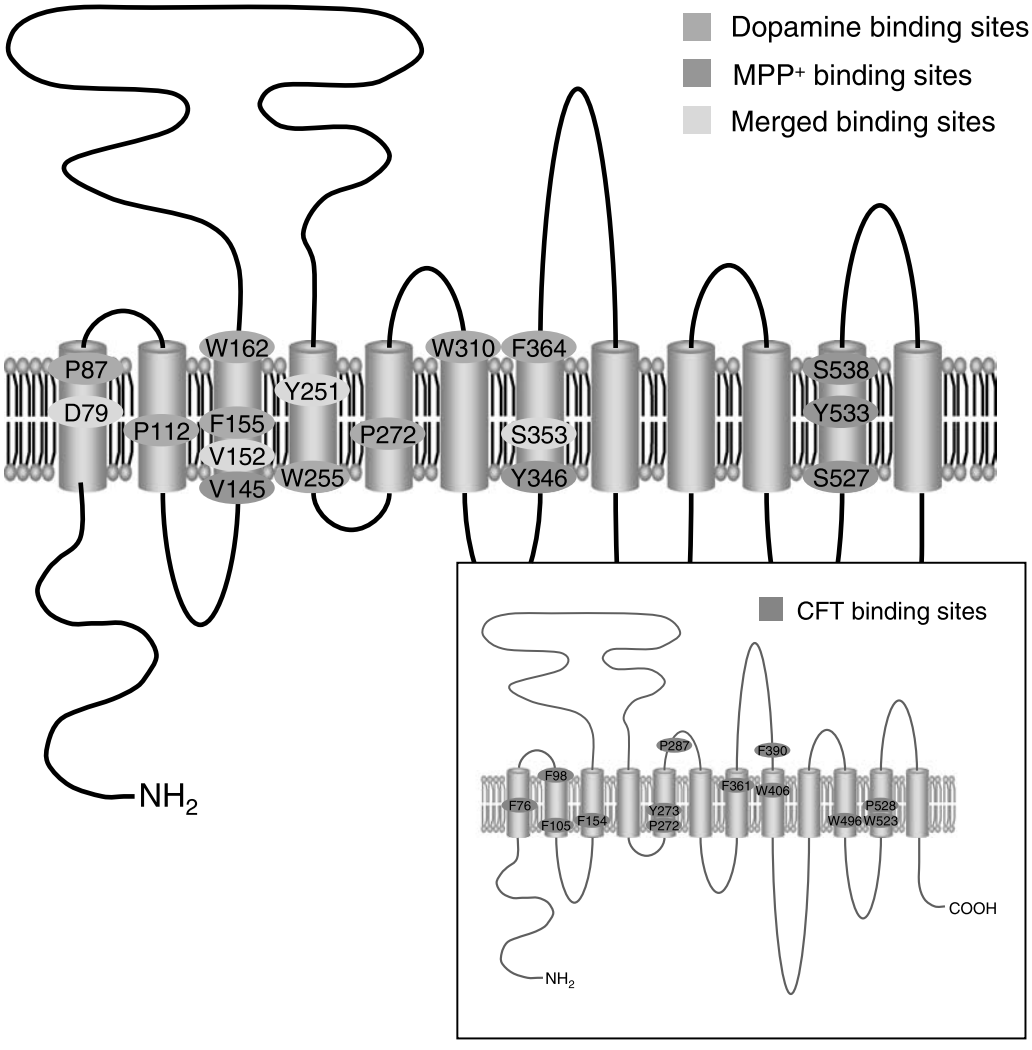

Fig. 1. Putative topology and structural features of the DAT protein. As suggested by the primary sequence of the cDNA, the protein has 12 transmembrane domains (TM) with both N- and C-termini located with the cytoplasm. Amino acids residues reported to be important for the affinities of dopamine, 1-methyl-4-phenylpyridinium (MPP^+) and cocaine derivative (−)-2β-carbomethoxy-3β-(4-fluorophenyl)-tropane (CFT; inset) at the DAT molecule are illustrated with colored boxes (labeling according to the sequence of the human DAT published by (Giros et al., 1992). The amino acid residues were identified using site-directed mutagenesis and subsequent binding/uptake studies (see text for details)

of mesencephalic DA neurons with most pronounced immunoreactivity in striatum and nucleus accumbens. The VTA and other brain areas with established DA circuitry, such as the hypothalamic neurons, were stained less intensively. In human brain samples, similar levels of DAT expression were detected in somatodendritic and axonal domains of most mesotelencephalic DA neurons (Miller et al., 1997; Ciliax et al., 1999).

The major physiological role of DAT is the termination of neurotransmission by rapid reuptake of DA from the synaptic cleft into presynaptic terminals,

and it is believed to control the intensity and duration of DAergic neurotransmission by setting the concentration of DA in the extracellular space. Depending on cellular conditions, DAT is able to transport DA bi-directionally, but at normal membrane potential and Na^+ gradient the inward transport is greater than the outward transport (Hitri et al., 1994). The function of DAT is regulated or influenced by several other cellular factors and proteins including the presynaptic protein α-synuclein (Kitayama et al., 1994; Huff et al., 1997; Lee et al., 2001a). Pharmacological characterization of cloned monoamine transporters expressed heterologously have confirmed substrate specificity for the respective neurotransmitter as shown earlier in synaptosomal preparations (for review, see Amara and Kuhar, 1993; Giros and Caron, 1993; Hitri et al., 1994). Subsequently, several highly specific DAT inhibitors could be established such as diphenyl-piperazine derivatives (for example GBR12935 or GBR12909). The binding site of DA at the transporter molecule was initially investigated using chimeric dopamine-norepinephrine transporters (Buck and Amara, 1994). It includes an amino terminal domain (TM1–TM3) and with lower significance TM10–TM11. These data were confirmed using site-directed mutagenesis and subsequent binding/uptake studies using the mutated transporters in mammalian expression systems (Kitayama et al., 1992b, 1993, 1996c, 1998; Mitsuhata et al., 1998; Lin et al., 1999, 2000a, b; Lee et al., 2000; Lin and Uhl, 2002). Mutations of several amino acid residues in TM1–7 could be identified to reduce binding affinity of DA at the DAT molecule (Fig. 1). On the other hand, the binding sites of the cocaine derivative (−)-2β-carbomethoxy-3β-(4-fluorophenyl)-tropane (CFT) were extensively characterized (Fig. 1; Kitayama et al., 1992b, 1996c; Lin et al., 1999, 2000a, b; Lee et al., 2000; Lin and Uhl, 2002, 2003). The displayed amino acid residues significantly influence the affinities of the respective substance, but from the site-directed mutagenesis experiments it is not clear whether all of these residues are located within the binding sites of the compounds or whether they affect the binding in an allosteric manner. However, some of the positions of amino acid residues lie close together while other amino acid residues are fare away from each other in the primary sequence. On the other hand, these residues could lie next to each other in the mature DAT structure even if they are not nearby in primary sequence. Interestingly, there are some mutations selectively alter the affinity of cocaine analogs, but spare their affinities for DA (compare Fig. 1 with inset; for review see Uhl and Lin, 2003).

In this review we discuss the importance of the DAT molecule for the selective vulnerability of dopaminergic cells against specific neurotoxins as well as in Parkinson's disease (PD). Thus, we summarize the data on the importance of the DAT molecule for the selectivity of neurotoxins such as 6-hydroxydopamine, 1-methyl-4-phenylpyridinium (MPP^+) and structurally related compounds towards DAT expressing cells. We include a discussion on structural requirements of such compounds for both cellular uptake by the DAT and DAT-mediated cytotoxicity. Since the DAT molecule is discussed to be involved in the selectivity of dopaminergic degeneration in PD, we compare these toxicological data with evidences for the importance of the DAT in the etiopathogenesis of PD.

Importance of the DAT for selective dopaminergic toxicity

Although DAT is selective for its respective neurotransmitter DA, it can also transport synthetic or natural structural analogues of the neurotransmitter. Should such compounds interact with vital intracellular functions or structures, their penetration into the cell via the DAT might have significant consequences. The importance of the DAT for the selectivity of toxicity of such structural analogues of DA will be discussed in detail in the following sections (please refer to Fig. 2 for chemical structures).

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 1-methyl-4-phenylpyridinium (MPP^+)

The potent exogenous neurotoxin MPTP produces a Parkinson-like syndrome in mammalians by causing a selective degeneration of DA neurons in the SNpc with eosinophilic inclusions (Langston et al., 1983). After entering the CNS, MPTP is metabolized largely in astrocytes by monoamine oxidase type B into 1-methyl-4-phenyl-1,2,3-dihydropyridinium ($MPDP^+$), which spontaneously undergoes oxidation to MPP^+ (Chiba et al., 1984; Przedborski et al., 2000). Russ and co-workers provided evidences that MPP^+ uses the extraneuronal monoamine transporter (EMT) to depart glial cells (Russ et al., 1996). Once inside the dopaminergic cell, MPP^+ induces a deleterious cascade of events include mitochondrial respiration deficit, oxidative stress, and energy failure (Di Monte et al., 1986; Ramsay and Singer, 1986; Kutty et al., 1991; Storch et al., 1999, 2000a; Przedborski et al., 2000). In recent studies it becomes evident that other proteins, such as wild-type and PD related mutant α-synucleins, modulate MPTP/MPP^+ toxicity towards DA neurons both *in vivo* and *in vitro* (Lee et al., 2001a; Dauer et al., 2002; Lehmensiek et al., 2002; Sidhu et al., 2004).

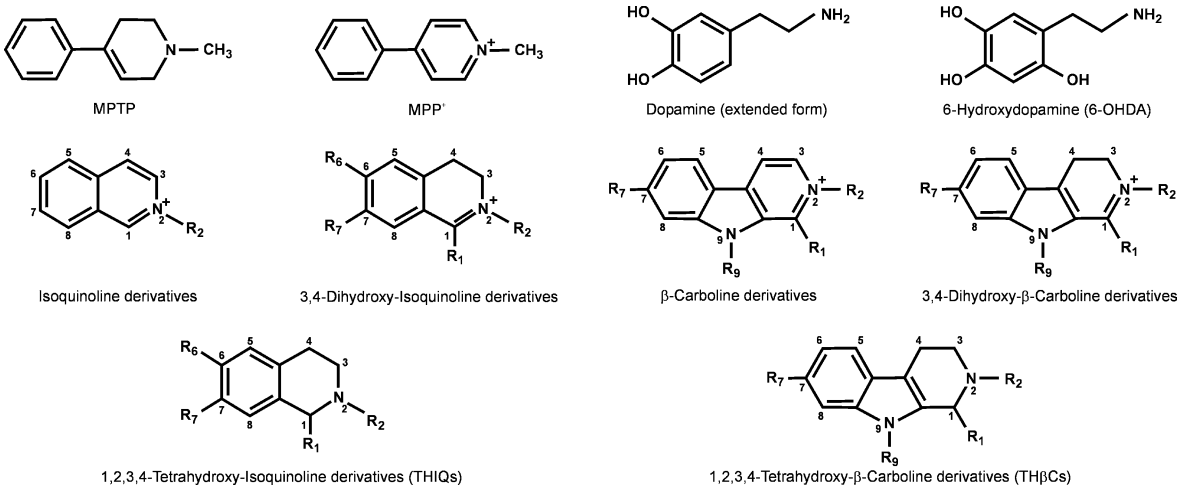

Fig. 2. Structures of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 1-methyl-4-phenylpyridinium (MPP^+), dopamine (extended form), 6-hydroxydopamine as well as isoquinoline and β-carboline derivatives

The selectivity of MPTP for DA neurons is considered to be a consequence of active uptake of its metabolite MPP^+ into DA neurons via the DAT (Chiba et al., 1985; Javitch et al., 1985). MPP^+ has high affinity for the DAT with K_m values of about 0.05 to 0.1 μM in rat synaptosomes, but 20 μM in cell lines with ectopic expression of DAT (Javitch et al., 1985; Pifl et al., 1993; Storch et al., 2004). It is taken up rapidly with V_{max} values comparable to those measured for DA. Cellular uptake of MPP^+ via the DAT shows a similar pharmacological profile compared to that of DA uptake. Consistently, the expression of DAT confers toxicity of low concentrations of MPTP/MPP^+ towards neuronal cells *in vivo* and *in vitro*. Mice lacking the DAT gene do not show MPTP-induced DAergic toxicity *in vivo* (Gainetdinov et al., 1997; Bezard et al., 1999), whereas mice with increased levels of DAT expression are more sensitive to MPTP (Donovan et al., 1999). Selective blockers of the DAT, such as mazindol, are reported to completely prevent MPTP-induced nigrostriatal toxicity, while inhibitors of the noradrenaline transporter fail to protect against MPTP toxicity (Javitch et al., 1985; Ricaurte et al., 1985; Mayer et al., 1986). Consistently, MPTP/MPP^+ show strong toxicity towards neuronal cell lines expressing the DAT (Kutty et al., 1991; Bloomquist et al., 1994; Storch et al., 2000a). These results were confirmed by toxicological studies on several neuronal and non-neuronal heterologous expression systems of the DAT (Kitayama et al., 1992a, 1998; Pifl et al., 1993, 1996; Storch et al., 1999). Using mutated DAT expressed in heterologous expression systems, Kitayama and co-workers demonstrated that sensitivity to MPP^+ toxicity correlates closely with the affinity (K_m value) of MPP^+ at the transporter, but does not correspond to uptake velocity (Kitayama et al., 1998). On the other hand, several studies report high uptake velocity or turn over number (V_{max}/maximal binding sites) of MPP^+ by the DAT, but low turn over number by the noradrenaline transporter compared to their natural substrates. In contrast, apparent affinity for MPP^+ at the noradrenaline transporter is higher than that at the DAT protein (Buck and Amara, 1994; Pifl et al., 1996). These latter data suggest that high V_{max} of MPP^+ by the DAT might contribute to the selectivity of DAergic toxicity of MPP^+.

Kitayama and co-workers demonstrated bi-directional transmembrane transport of MPP^+ through the DAT (Kitayama et al., 1993, 1996a). Using mutant DAT with different transport properties ectopically expressed in COS cells, Kitayama and co-workers (1998) showed that indeed the ratio of influx/efflux turnover through the transporter may be an important factor for MPP^+ toxicity (Kitayama et al., 1998). Some mutations of the DAT protein resulting in enhanced reverse transport compared to that of wild type DAT (Y273A; double mutant S353A/S335A; Y533F) leads to decreased sensitivity of the cells against MPP^+ toxicity (Kitayama et al., 1998). The binding site of MPP^+ at the transporter molecule was initially investigated using chimeric dopamine-norepinephrine transporters (Buck and Amara, 1994). In contrast to DA, not only the amino terminal domain spanning TM1–TM3, but also a domain including TM10–TM11 significantly influences apparent affinity of MPP^+ at the DAT. The above mentioned site-directed mutagenesis approach confirmed these data (see Fig. 1 for details; Kitayama et al., 1993, 1998; Mitsuhata et al., 1998; Lin et al., 1999; Lee et al., 2000). Dopamine and MPP^+ probably

recognize overlapping, but different binding sites at the DAT molecule. Taken together, these data clearly demonstrate that the DAT is responsible for the high sensitivity and selectivity of MPTP/MPP^+ toxicity towards DA neurons both *in vitro* and *in vivo*. However, it remains unclear whether affinity of MPP^+ at the transporter or turn over number is more important for cellular sensitivity to MPTP/MPP^+ toxicity. Recent studies demonstrated that mutant α-synucleins related to PD further increase DAT-mediated selectivity of MPP^+ toxicity, most likely by direct functional coupling to the DAT molecule (Lee et al., 2001b; Lehmensiek et al., 2002).

Pyridine derivatives structurally related to MPTP/MPP^+

Michel and co-workers (1989) studied the toxic effects of 13 potential environmental pyridinium compounds structurally related to MPTP/MPP^+ on mesencephalic DA neurons from rat *in vitro* (Michel et al., 1989). Among the group of these pyridinium compounds, only two very closely related compounds (2-methyl-MPP^+ and *p*-amino-MPP^+) showed selective effects against DA neurons, but were less effective and less selective compared to MPP^+. There are no uptake studies available with these substances. Haloperidol, a widely used antipsychotic compound with a pyridine structure is metabolized to haloperidol pyridinium ion (HPP^+) by cytochrome P450, a reaction similar to the conversion of MPTP to MPP^+. It has been proposed that long-term extrapyramidal symptoms, such as tardive dyskinesia, could be caused by neurotoxic effects of HPP^+ or other metabolites of haloperidol following uptake by monoamine transporters. Indeed, HPP^+ and other metabolites are shown to be toxic *in vitro* to rat mesencephalic cultures and neuroblastoma cells (Bloomquist et al., 1994). Uptake studies on DAT, NET and SERT using transfected COS-7 cells and mouse synaptosomal preparations revealed that haloperidol, HPP^+ and their tetrahydropyridinium analogs inhibit all monoamine transporters without relevant differences of potencies (Bryan-Lluka et al., 1999). Accordingly, using heterologous expression systems we were not able to show DAT-dependent toxicity of HPP^+ (unpublished results). These data suggest that haloperidol and its analogs are not likely to cause neurotoxicity by a mechanism similar to that of MPTP/MPP^+ involving the uptake by monoamine transporters.

Isoquinoline derivatives

Isoquinoline derivatives refer to isoquinoline (IQ) itself, various reduced species, like 3,4-dihydro-IQs and 1,2,3,4-tetrahydroisoquinoline (TIQ), and their substituted congeners (e.g. 1-benzyl-TIQ). They are widely distributed in the environment and occur naturally in mammalian brain where they are synthesized by an enzymatic and non-enzymatic Pictet-Spengler condensation of biogenic amines with aldehydes (Melchior and Collins, 1982). IQs are metabolized by cytochrome P_{450}, *N*-methyltransferases and monoamine oxidases leading to charged quaternary forms (isoquinolinium cations; McNaught et al., 1998). Interestingly, there are evidences for altered levels of DA-derived catecholic IQs, in particular derivatives of 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (salsolinol) and the synthesizing enzymes in patients with PD

(Maruyama et al., 1996; Naoi et al., 1998). Since many IQ derivatives are structurally related to MPTP/MPP^+ (refer to Fig. 2), IQs are considered as endogenous and/or exogenous DAergic neurotoxins playing a role in the etiopathogenesis of PD (McNaught et al., 1998).

The partial selectivity of IQ derivatives towards DAergic cells is considered to be a consequence of cellular uptake by the DAT as shown for MPP^+. Indeed, several IQ derivatives show partially selective but weak cytotoxicity in DAergic cells lines and primary DA neurons (McNaught et al., 1996a; Goto et al., 1997; Maruyama et al., 1997; Takahashi et al., 1997; Storch et al., 2000a). *In vivo* studies using experimental animals including non-human primates demonstrated that some IQs are able to produce a parkinsonian syndrome with neurochemical, histological and behavioural changes after chronic treatment (for review, see McNaught et al., 1998). To confirm the importance of the DAT as well as the structural requirements for DAT-mediated toxicity induced by IQ derivatives we used non-neuronal and neuronal heterologous expression systems of DAT, which are extremely sensitive to very low concentrations of MPP^+ (Storch et al., 1999, 2002). Out of 21 IQ derivatives, only the 2[*N*]-methylated compounds showed enhanced cytotoxicity in both DAT expressing cell lines with 2 to 14-fold reduction of half-maximal toxic concentration (TC_{50} values) compared to parental cell lines. The rank order of selectivity in both cell systems was: $MPP^+ \gg 2[N]$-methyl-$IQ^+ > 2[N]$-methyl-norsalsolinol (2[*N*]-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline) = 2[*N*]-methyl-salsolinol. The selective toxicity of these compounds can be blocked by DAT inhibitors, such as GBR12909.

Neutral and quaternary IQs are able to partially inhibit [^{3}H]DA accumulation into striatal synaptosomes from rat, but they are far less potent than MPP^+ (IC_{50} values in the micro- to millimolar range versus less than 1 μM; (Heikkila et al., 1971; Hirata et al., 1990; McNaught et al., 1996b). The most potent blockers of [^{3}H]DA uptake are TIQ derivatives with catechol structures. Using tritiated substances, Heikkila and co-workers showed accumulation of 6,7-dihydroxy-1,2,3,4-TIQ and 1-methyl-4,6,7-trihydroxy-1,2,3,4-TIQ in rat striatal synaptosomes (Heikkila et al., 1971). Takahashi and co-workers studies the uptake characteristics of catecholic IQs in SH-SY5Y cells using a HPLC-based method (Takahashi et al., 1994). They found that only the (*R*)-isomer of 2[*N*]-methyl-salsolinol is transported by the DAT, while its (S)-isomer, salsolinol and 1,2-dimethyl-6,7-dihydroxyisoquinolinium are not. DA and other DAT inhibitors block the uptake of 2[*N*]-methyl-(*R*)-salsolinol. Similarly, 2[*N*]-methyl-IQ^+ has been shown to accumulate in rat striatal synaptosomes (Hirata et al., 1990). Another extensive study using rat striatal synaptosomes revealed that 2[*N*]-methyl-IQ^+, (*R*)-salsolinol and 2[*N*]-methyl-(*R*)-salsolinol are accumulated via the DAT (Matsubara et al., 1998b). Quaternization of TIQ's reduced their original properties as substrates for the DAT. Overall the data discussed here demonstrate that several IQs, in particular 2[*N*]-methylated catecholic IQs, are substrates for the DAT with both moderate to low affinities at the DAT and moderate uptake velocities compared to MPP^+ and DA.

We suggest that 2[*N*]-methylated IQ derivatives structurally related to MPTP/MPP^+ are selectively toxic to DA neurons via uptake by the DAT.

However, low affinity and uptake velocity of IQs at the DAT may be a rate-limiting factor for DAergic toxicity. Future studies are warranted to compare uptake kinetics and toxicity of IQs mediated by other monoamine transporters to further confirm the role of DAT-mediated uptake for their DAergic toxicity (Buck and Amara, 1994; Pifl et al., 1996).

β-Carboline derivatives

β-Carbolines (βCs; pyrido-indoles) are heterocyclic molecules occurring both exogenously and endogenously (for review, see Collins and Neafsey, 2000), which are structurally related to the parkinsonian neurotoxin MPTP/MPP^+ (for structures refer to Fig. 2). Some of these βCs are reported to cause nigrostriatal neurodegeneration and behavioral impairment in several animals, including non-human primates (Collins et al., 1986; Matsubara et al., 1998a), most likely by sequential biotransformation via *N*-methylations to β-carbolinium ions (Waring et al., 1989; Collins et al., 1996; Collins and Neafsey, 2000). These data are consistent with *in vitro* studies showing cytotoxicity of *N*-methylated β-carbolinium ions in primary mesencephalic cultures from rat with partial selectivity for DA neurons (Collins et al., 1996; Matsubara et al., 1998a). In HEK-293 cells expressing the DAT, only 2[*N*]-methylated compounds showed enhanced cytotoxicity with 1.3 to 4.5-fold reduction of TC_{50} values compared to the parental cell line (Storch et al., 2004). The rank order of selectivity was: $MPP^+ \gg$ 2[*N*],9[*N*]-DiMe-harminium > 2[*N*]-Me-harminium > 2[*N*],9[*N*]-DiMe-harmanium = 2[*N*]-Me-norharmanium > 2[*N*]-Me-harmanium > 2[*N*],9[*N*]-DiMe-norharminium. These results in HEK-hDAT cells exposed to 2[*N*]-methylated and 2[*N*],9[*N*]-dimethylated βCs parallel the toxicity of the same compounds towards DA neurons in fetal rat mesencephalic cultures (Cobuzzi et al., 1994; Collins et al., 1995, 1996; Matsubara et al., 1998a).

As for MPP^+, cellular uptake of βCs or rather β-cabolinium ions by the DAT is considered to be involved in the selectivity of DAergic toxicity of these compounds. Indeed, Drucker and co-workers (1989) showed that 2[*N*]-methylated β-carbolinium ions are able to inhibit [^{3}H]DA uptake into rat striatal synaptosomes, but IC_{50} values are lower compared to MPP^+ (12 to approx. 150 μM for β-carbolinium ions and 0.5 μM for MPP^+, respectively; Drucker et al., 1990). Furthermore, the partially competitive nature of inhibition two effective compounds, 2-methyl-harmine and harmine, indicate that uptake of the β-carbolines is mediated in part by synaptosomal DAT (Drucker et al., 1990). This view is further supported by the fact that accumulation of 2-[^{14}C]methyl-harmane into synaptosomes is partially blocked by the potent DAT inhibitor nomifensine (Drucker et al., 1990). Using the same experimental setup, Matsubara and co-workers measured the uptake of the neutral β-carboline norharmane and quaternary β-carboliniums 2-methyl-norharmanium and 2,9-dimethyl-norharmanium, respectively, using a HPLC-based method (Matsubara et al., 1998b). They found that norharmane is an unfavorable substrate for the DAT, while the *N*-methylated compounds showed DAT mediated influx into striatal synaptosomes with 1–4 times higher K_m values and ≈10 times lower uptake velocity (V_{max}). We established a novel uptake assay in HEK-293

cells expressing the DAT utilizing the fluorescent properties of βCs (Storch et al., 2004). Out of 12 non-methylated and methylated βC derivatives (refer to Fig. 2 for structures) all 2[*N*]-methylated βCs were transported into the cell through the DAT with up to 5 times higher K_m and 12 to 220 times lower V_{max} values compared to DA and MPP^+ (Storch et al., 2004). Compared to the toxicity data in the same cell line, there is a weak correlation of DAT-mediated selectivity with the affinity of βCs at the DAT (K_m values), but not with V_{max}. These data suggest that DAT-mediated cellular uptake of 2[*N*]-methylated βCs is most likely responsible for their selective toxicity towards DA neurons.

The halogenated β-carboline derivative 1-trichloromethyl-1,2,3,4-tetrahydro-β-carboline (TaClo) is formed under physiological conditions via the Pictet-Spengler condensation from the biogenic amine tryptamine and the synthetic aldehyde chloral (Bringmann et al., 1995). Indeed, several studies provide evidences for monoaminergic degeneration *in vivo* (Bringmann et al., 1995; Gerlach et al., 1998). TaClo is able to inhibit DA and serotonin uptake with IC_{50} values in the μM range. Furthermore, *in vitro* toxicological studies using DAergic and serotoninergic cell lines (IMR-32 and JAR, respectively) revealed that TaClo penetrates cell membranes by passive diffusion and not by selective uptake systems (Bringmann et al., 2000). Consistently, there is no DAT-mediated toxicity and uptake of TaClo or its *N*-methylated derivative in HEK-293 cells expressing DAT (unpublished observations).

6-Hydroxydopamine

6-hydroxydopamine (6-OHDA) is a toxin selective for catecholaminergic cells *in vivo*, which is widely used to produce animal models of PD (Breese and Traylor, 1970; Jonsson and Sachs, 1971; Kostrzewa and Jacobowitz, 1974). 6-OHDA can be formed from DA by non-enzymatic hydroxylation in presence of Fe^{2+} and H_2O_2. Moreover, Andrew et al. (1993) recently detected endogenous 6-OHDA in urine samples of patients suffering from PD, suggesting that this compound may be involved in the pathogenesis of PD (Andrew et al., 1993). The partial selectivity of 6-OHDA toxicity *in vivo* towards catecholaminergic neurons was attributed to its uptake via catecholamine transporter systems, including the DAT (Jonsson and Sachs, 1971; Kostrzewa and Jacobowitz, 1974). Furthermore, Cerruti and co-workers (1993) showed partial protection against 6-OHDA toxicity by selective and potent DAT inhibitors in cultured DA neurons (Cerruti et al., 1993a). On the other hand, several studies using different blockers of monoamine transporters as well as heterologous expression systems of the DAT suggest that 6-OHDA toxicity *in vitro* is not mediated by transmembrane uptake via catecholaminergic uptake systems (Abad et al., 1995; Storch et al., 2000b). Furthermore, *in vitro* studies using different catecholaminergic and non-catecholaminergic primary cell and cell lines revealed no selectivity of 6-OHDA-induced toxicity for catecholaminergic cells (Michel et al., 1990; Abad et al., 1995; Storch et al., 2000b). 6-OHDA shows only low affinity at the DAT (Michel et al., 1990; Decker et al., 1993) and no detectable

levels of 6-OHDA were found in cytosolic extracts of PC12 or SH-SY5Y cells following incubation with up to 600 μM 6-OHDA (Decker et al., 1993). The reasons for the discrepancies between the *in vivo* and *in vitro* experiments remain unclear.

Structural requirements for DAT-mediated uptake and toxicity

The herein reviewed studies on DAT-mediated toxicity and cellular uptake of azaheterocyclic molecules provide significant information on structure-activity/toxicity (selectivity) relationships. The intermolecular distance between the *N*-atom and the centrinoid of the benzene or catechol ring is one of the most important factors for the affinity of compounds at the DAT molecule. The conformation of the DA molecule at the uptake site of the DAT is the extended or *trans* form (Horn, 1974; Meiergerd and Schenk, 1994). The distance between the *N*-atom and the centrinoid in MPP^+ and β-carboline derivatives is very close to that of the extended DA conformation. In contrast, this distance is much shorter in IQ derivatives, which is most likely responsible for their lower affinity and uptake velocity at the DAT and subsequent lower selectivity of toxicity as compared to MPP^+ and βC derivatives (Storch et al., 2002, 2004). The charge of the *N*-atom seems to be another factor not only for the affinity of the compounds to the transporter molecule, but also for the transmembrane transport of the compounds by the DAT (Matsubara et al., 1998b). Indeed, DAT-mediated toxicity and uptake were restricted to quaternary 2[*N*]-methylated compounds with a net charge comparable to DA, whereas structurally identical neutral compounds lack selective toxicity towards DAT-expressing cell lines (see Fig. 2). Thus, 2[*N*]-methyl-IQ^+, which has the closest structure-based relationship to MPP^+ within the IQ derivatives (no side chains; equivalent charge of the *N*-atom of the pyridine ring), showed the highest selectivity towards DAT-expressing cells. All modifications of the original structure of 2[*N*]-methyl-IQ^+ resulted in partial or complete loss of selective toxicity towards DAT-expressing cells. Among the *N*-methylated IQ derivatives, catecholic fully oxidized IQs (TIQ derivatives) showed less selectivity for DAT-expressing cells compared to 2[*N*]-Me-IQ^+. In agreement with Kawai and co-workers (Kawai et al., 1998), methoxylation of carbonyl residues 6 and 7 of the benzene ring, as in 2[*N*]-Me-norsalsolidine, completely abolished DAT-mediated toxicity. While methylation at position 1 of the pyridine ring does not alter toxicity/selectivity for DAT-expressing cells, large side chains at this position seems to inhibit DAT-dependent toxicity. In *N*-methylated βC derivatives, an addition of a methoxy group on position 7 of the aromatic ring increases the DAT-mediated toxicity, but not their uptake kinetics at the DAT molecule. Systematic comparison of DAT-mediated toxicity induced by βCs with their uptake kinetics suggests that the affinity of azaheterocyclic compounds at the DAT is the major factor for their toxic selectivity towards DAT-expressing cells. Thus, the lower affinities (greater K_m values) of βCs and IQs at the DAT compared to that of MPP^+ might explain the moderate selectivity of these compounds towards DA neurons.

Importance of DAT in the etiopathogenesis of Parkinson's disease

MPTP produces a Parkinson-like syndrome in human and non-human primates with remarkable similarities to PD (Langston et al., 1983; Forno et al., 1986). Langston and co-workers recently reported evidences of active neurodegeneration in the SNpc in humans years after MPTP exposure in humans, suggesting that MPTP intoxication may lead to a progressive disorder of the nigrostriatal pathway (Langston et al., 1999). As described above, there are many exogenous and endogenous compounds structurally related to MPTP/MPP^+ showing toxicity in the central DAergic system, leading in part to parkinsonian syndromes. This large body of evidences for the pivotal role of the DAT in selective vulnerability of DA neurons against parkinsonism-inducing compounds leads to extensive morphological and genetic investigations on the importance of the DAT molecule in PD.

Morphological studies

Three groups of DA neurons in different regions show substantially different extent of neuronal cell loss in PD brains. DA neurons of the arcuate nucleus of the hypothalamus providing the tuberoinfundibular DAergic system display no detectable loss in PD. DA neurons of the VTA those provide DAergic innervation of the cerebral cortex and limbic forebrain show only 40–60% cell death in PD brains (Uhl et al., 1985; German et al., 1989). On the other hand, DA neurons within the SNpc providing the dense DAergic projections to the striatum display the highest degree of degeneration in PD brains (Waters et al., 1988; Pakkenberg et al., 1991). Consistently, DA uptake sites have been found to be markedly decreased in the striatum in patients with PD both postmortem and *in vivo* (Janowsky et al., 1987; Leenders et al., 1990; Miller et al., 1997; Tatsch et al., 1997). These data document a strong correlation between the density of DAT expression and the extent of DA cell losses in PD brains (SNpc > VTA > arcuate nucleus in both cases). There are some intact DA neurons that lack Lewy body pathology in the SNpc from PD patients showing average DAT mRNA levels lower than those of age- and gender-matched controls (Uhl et al., 1994). DAT studies in living humans using PET or SPECT combined with autoradiographic studies of the DAT in tissue samples obtained post mortem suggest that there is a significant individual variation in the DAT expression, which is not related to age known to influence the DAergic system (Staley et al., 1995): Subjects of similar age can display up to 2-fold differences in radioligand binding to the DAT. These differences are in the same range as those that can significantly alter the susceptibility to DAergic toxins in many transgenic mice models of the DAT (Donovan et al., 1999). However, these morphological data suggest that DAT-based microcompartmentalization of DA or other toxins in DA neurons may be involved in selective neurodegeneration in PD.

Molecular biology and genetic studies

Studies of the large human gene for the DAT on the short arm of chromosome 5 (5p 15.3) reveal several polymorphisms (Giros et al., 1992; Sano et al., 1993; Morino et al., 2000). Studies of these polymorphisms could identify allelic gene

variants that could contribute to genetically determined human individual differences in both protein coding sequences and levels of DAT expression. These individual differences could provide plausible sources for differential vulnerabilities to DAergic toxicities. However, association studies of these polymorphisms with PD revealed conflicting results: Two independent research groups demonstrated a significant association of a single nucleotide polymorphism in exon 9 (1215A/G) with PD (Morino et al., 2000; Nishimura et al., 2002), but two other groups were not able to confirm these results (Kimura et al., 2001; Lin et al., 2002). The DAT gene contains a 40 base pair variable number tandem repeat (VNTR) in the 3' untranslated region of the gene (Sano et al., 1993). Le Couteur and co-workers (1997) reported an association of this DAT gene VNTR with PD in Caucasians (Le Couteur et al., 1997), but not in a Chinese population (Leighton et al., 1997; Lin et al., 2002): The rare 11-copy allele of the DAT VNTR (0.3% frequency of all alleles) increases the risk of PD about 10-fold in Caucasians (Le Couteur et al., 1997). This result is of some interest, because the DAT VNTR sequence includes possible binding regions for several transcription factors, and indeed VNTR polymorphism effects translation of the DAT protein *in vivo* (Heinz et al., 2000). However, such an increase in the 11-allele of the DAT gene was not observed in two other studies in caucasian populations (Plante-Bordeneuve et al., 1997; Mercier et al., 1999). Interestingly, the homozygote 10-copy genotype of variable number tandem repeat dopamine transporter gene might confer protection against PD for male, but not to female patients (Lin et al., 2003). The reasons for these differences are unclear, but most likely occurred as a result of methodological problems such as selection bias, false positive results because of small study populations and/or multiple testing without adjustment. Furthermore, geographic variations in toxins and toxin exposure, variations in metabolic genotypes between the populations might be responsible for different results in the genetic studies. Together, the association studies concerning the DAT gene and the risk of PD did not reveal conclusive results.

Conclusions

The *in vivo* and *in vitro* data show that the DAT plays a pivotal role for the selective DAergic toxicity of endogenous and exogenous *N*-methylated heterocyclic molecules structurally related to MPTP or its active metabolite MPP^+ and that the DAT may be important for selective degeneration of the DA neurons found in PD. Epidemiological studies indicate that a number of environmental factors increase the risk of developing PD. However, no specific toxin has been found in the brain of PD patients, and in many instances the parkinsonism produced by toxins is not that of typical Lewy body PD. Nevertheless, recent findings of altered levels of potential DAergic neurotoxins, such as IQ derivatives, in patients with PD together with the finding that the level of degeneration in PD closely corresponds with the level of cellular DAT expression further support the hypothesis that miscompartmentalization of toxic compounds by the DAT might be responsible for the selectivity of DAergic degeneration found in PD. However, genetic association studies on the DAT

gene in PD did not reveal conclusive results and future studies have to clarify the importance of interactions of the DAT gene or alterations of function/expression of the DAT protein with environmental factors for the pathogenesis of PD. The identification of a DAT-based mechanism that explains selective DA cell loss in PD could have several implications: Compounds that inhibit intracellular uptake mediated by DAT would be of interest for therapeutic trials targeted towards slowing the disease onset and/or progression. Such agents could modulate transporter function, for example by altering the phosphorylation of DAT (Kitayama et al., 1994; Huff et al., 1997), or act directly on the DAT molecule. As demonstrated above, DA and MPP^+ probably recognize overlapping, but different binding sites at the DAT molecule, suggesting a possible strategy for developing drugs which specifically block uptake or stimulate release of potential DAergic neurotoxins via the DAT without affecting DA uptake (as demonstrated already for cocaine derivatives and other DAT blockers, showing differential effects on the release of MPP^+ and DA, respectively, through the DAT (Kitayama et al., 1996b). Furthermore, individual differences of DAT expression, genetically or environmentally determined, could serve as markers for susceptibility for PD.

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