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The functional domains of dopamine transporter for cocaine analog, CFT binding

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Abstract

Cocaine analogue, CFT (2β-carbomethoxy-3β-(4-fluorophenyl) tropane) binding to dopamine transporter (DAT) in different species is quite heterogeneous. CFT is scarcely detected in bovine DAT whereas it is conspicuous in humans. To examine the structural basis for this functional discrepancy, we analyzed transporter chimeras of these two DATs. The CFT binding activities are avid in all of the chimeric DATs of which both of the 3rd and the 6-8th transmembrane domain (TM) are composed of human DAT sequences. On the contrary, CFT binding activities were scarcely detected if either or both of two regions are replaced with bovine sequences. These findings indicate that the CFT binding absolutely requires human DAT sequences, at least, in the regions encompassing the 3rd and 6-8th transmembrane domain (TM), and that these regions might contribute to form the 3-dimensional pocket for CFT binding.

Keywords: dopamine transporter (DAT), cocaine, cocaine binding, CFT, CFT binding, transmembrane domain

Introduction

Dopaminergic neurotransmission is terminated by the activity of the presynaptic dopamine transporter (DAT) that mediates the reuptake of extracellular dopamine (DA) into the cell, and such a re-uptake is a major regulatory process of controlling signal transmission by monoamine neurotransmitters including dopamine, norepinephrine and serotonin (Horn, 1990; Shimada *et al*., 1991; Iversen, 1992; Uhl, 1992; Uhl and Hartig, 1992; Sora *et al*., 2001). DAT also represents important target for the psychostimulants such as tricyclic antidepressants and cocaine. Cocaine specifically binds to DAT and inhibits DA re-uptake, thereby increasing extracellular DA concentrations and enhancing DA receptor occupancy (Snyder and DAmato, 1986; Shimada *et al*., 1991). In self administration studies, the potencies of cocaine and related drugs in inhibiting the binding of [^{3}H]Mazindol, a DA analog, to DAT are closely correlated with their reinforcing properties. Based on these studies, the augmented dopaminergic neurotransmission by inhibiting the reuptake system represents the best current explanation of euphoria, reward, and reinforcement of cocaine (Kuhar *et al*., 1991).

The determination of the DAT structures contributing to cocaine analog, CFT (2β-carbomethoxy-3β-(4-fluorophenyl) tropane) binding would be prerequisite for providing tools for treating cocaine overdose and, possibly, cocaine abuse. However, although approaches to identify the structural domains responsible for the DAT functions have been performed, the discrete site for CFT binding is still to be determined. We previously reported that CFT binding is scarcely detected in the cells expressing bovine DAT (bDAT), whereas it is avid in the cells expressing human DAT (hDAT) (Lee *et al*., 1996b). The primary sequences of human and bovine DAT are most similar, having an 89% overall identity in their amino acid sequences, and hydropathy analyses of these sequences indicate the identical putative topology of 12 transmembrane domain (TMD) which places the N- and C- termini intracellularly, three or four of the glycosylation sites extracellularly, and several consensus phosphorylation sites intracellularly. These distinctively distinguished properties of two DAT proteins can provide a cue to delineate the structure-and-function relationship. In the present studies, functional analysis of the chimeras between human and bovine DATs reveals the domains that are involved in the specific function for the CFT binding: both the 3rd transmembrane domain (TM) and the 6th-8th TM in DAT are absolutely required for the CFT binding.

Materials and Methods

Construction of DAT chimeras

The molecular cloning reveals 12 putative TM topology of DAT and restriction enzyme recognition sites of their

Table 1. Both of the opposite half of human DAT sequence are needed for CFT binding

		CFT binding (% hDAT)
hDAT		100.0±11.4
bDAT		2.6±4.2
Chi 1		ND
Chi 2		12.1±5.4

Neither of the chimera 1 and 2 did not show avid CFT binding. It suggests CFT binds to the DAT through more than one domain, each of them located in the opposite half. The values are means ± SEM of six independent experiments performed in duplicate. ND, not detected

Table 2. The 3rd TM is essential for the CFT binding in the 1st half of human DAT

		3rd TMD	CFT binding (% hDAT)
hDAT		■	100.0±11.4
Chi 2		□	12.1±5.4
Chi 3		□	19.9±4.9
Chi 4		■	73.3±13.0
Chi 5		□	3.3±3.0
Chi 6		□	11.2±9.7

The first half of DAT is divided into the segments encompassing the 1st-2nd TMD, the 3rd TMD, and the 4-5th TMD using the restriction enzyme sequences such as *Bgl*II at the 398th nucelotide, and *Sca*I at the 644th nucleotide. Each bovine segment in the first half of the chimera 2 was returned back to that of human segment, and then the CFT binding activities were analyzed. The return of the 3rd TM with human sequence recovered the CFT binding activity markedly, whereas those of 1-2nd and 4-5th TM didn't. These findings suggest that the human sequence of the 3rd TM is an essential domain in the first half of DAT. The values are means ± SEM of three to six independent experiments performed in duplicate.

cDNA (Snyder *et al*., 1986; Uhl, 1990; Shimada *et al.*, 1991; Usdin *et al*., 1991). Unique restriction sites in the cDNA of the human DAT (*Bgl*II, *Sca*I, *Cla*I, *Pst*I at nucleotides 399, 644, 1303, and 1743, respectively) were used for the construction of chimeric cDNAs. Because the *Bgl*II and *Sca*I recognition sites on the bovine DAT are also unique and are located at identical positions, these sequences could be applied to the construction of chimeric cDNAs by the restriction-ligation reaction (Table 1, 2). By contrast, *Cla*I and *Pst*I sites do not exist at the equivalent positions, so the bovine DAT cDNA fragments with the *Cla*I (or*Pst*I) site at its end were obtained by PCR, then substituted into hDAT for the construction of chimera such as 5, and 6. In addition, *Eco*RI restriction recognition sequences, which do not exist in both of the human and bovine DAT cDNA, were engineered at nucleotide 924 of each human and bovine DAT cDNA by site-directed mutagenesis using the pALTER-1 system (Promega Co., Madison, WI, USA) and, then, used for the construction of chimeric cDNAs. Even though the alteration of amino acid (from alanine to phenylalanine) by engineering *Eco*RI recognition sequence didn't cause any effect on the DAT functions, this sequence was transformed back to the original sequence by site-directed mutagenesis described above after the chimera construction to avoid any kind of possible effects by introducing the *Eco*RI recognition sequence. The nucleotide sequences of chimeric cDNAs were confirmed by dideoxy-sequencing using the Cy5™ Autoread™ automatic sequencing system (Pharmacia Biotech, Uppsala, Sweden).

Functional assay of DAT

Each cDNA of the wild-type human DAT, bovine DAT, and chimeric DAT was subcloned into a mammalian expression vector, pCDNA3.1(+) (Invitrogen Co., San Diego, CA, USA) and then introduced into COS-1 cells using FuGENE™6 transfection reagent (Boehringer Mannheim Co., Mannheim, Germany). The cells were plated on 24-well plates with the number of 3×10^4 cells per well, and cultured overnight in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum. DNA (0.5 μg/well) was mixed with FuGENE™6 reagent (0.7 μl/well), then incubated for 15 min at room temperature. The DNA-FuGENE™6 complex was added to the cells. After incubation for two days, the tritium labeled cocaine analog 2β-carbomethoxy-3β-(4-fluorophenyl) tropane ([^{3}H]CFT, also designated [^{3}H]WIN35,428) binding assays were performed in a modified Krebs-Ringer HEPES (KRH) buffer containing 25 mM HEPES (pH 7.4), 125 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5.6 mM glucose, 1 mM sodium ascorbate (for [^{3}H]DA uptake, 10 μM pargyline was added). Cells were washed with the modified KRH buffer, and then incubated with 20 nM [^{3}H]DA (51 Ci/mmol, Amersham Co., Buckinghamshire, UK) or 10 nM [^{3}H]MPP$^+$ (83 Ci/mmol, Dupont-New England Nuclear Co., Boston, MA, USA) for 10 min at 37°C. Uptake reactions were terminated by adding ice-cold KRH buffer and the cells were washed with the same buffer three times. The cells were solubilized with 0.5 M NaOH and their radioactivities were analyzed by liquid scintillation spectroscopy. The binding of [^{3}H]CFT to DATs was determined by incubating cells with the KRH buffer containing 10 nM [^{3}H]CFT (87 Ci/mmol, Dupont-New England Nuclear Co.) for 2 h at 4°C. Specific uptake or binding were defined by subtracting the uptake (or binding) of the cells transfected with pCDNA3.1 vector, in the identical conditions, from the respective uptake or binding obtained from the hDAT, bDAT or chimeric DAT transfectants.

Results

Human sequences of the 3rd and 6-8th TM are required for the CFT binding

Table 3. The 6-8th TM is the other essential structure for CFT binding

DAT		6-8th TMD	CFT binding
hDAT			100.0±11.4
Chi 7			ND
Chi 8			119.9±7.4
Chi 9			70.2±10.0

The last half of DAT can be divided into the 6-8th TM, the 9-12th TM and the carboxyl terminal tail. In human DAT, each 3 segments was replaced with the respective one of bovine DAT to find out segment(s) responsible for the CFT binding in the last half of DAT. The substitution of 9-12th segment didn't abolish and rather increased the CFT binding activity by 20%. However, the CFT binding activity was completely disappeared by substituting the segment encompassing the 6-8th TM (in chimera 7), suggesting the 6-8th TM is another structure required for the CFT binding. The substitution of the carboxyl terminal tail (in chimera 9) decreased the CFT binding but not drastic like that of the 6-8th TM. The values are means ± SEM of four to six independent experiments performed in duplicate. ND, not detected.

The CFT binding is hardly detected in bovine DAT whereas it is avid in human DAT. This finding enables us to construct the chimeras of these two DATs to find out the structures responsible for CFT binding. At first, we substituted the first (the amino terminal - the 5th TMD) or second half (the 6th TMD - the carboxyl terminal) of DAT with the equivalent portion of the counterparts. The chimera 1 and 2, in which one half include bovine DAT, shows no or scanty activities for CFT bindings, suggesting both of the opposite half of human sequences are required for CFT binding (Table 1).

Based on the *Bgl*II and *Sca*I restriction enzyme recognition sequences, the first half of bovine DAT cDNA can be divided into 3 segments, containing the 1st-2nd TM, 3rd TM, and 4-5th TM. Each bovine segment in the first half of the chimera 2 was returned back to that of the human segment, and then the CFT binding activities were analyzed. The return of the 3rd TM with human sequence recovered the CFT binding activity to 73.3 ± 13.0% of human DAT (see chimera 4 in Table 2), whereas the return of the 1st-2nd or 4-5th TM with human DAT didn't show any significant recovery of the CFT binding (see chimera 3 and 5 in Table 2). Moreover, the substitution of the 3rd TM in the human DAT with the bovine sequence almost completely abolished the CFT binding activities (see chimera 6 in Table 2). These results strongly suggest that the human sequence of the 3rd TM is an essential segment for the CFT binding. However, the substitution of the bovine sequence of the 3rd TM into human DAT (in chimera 10) didn't induce the CFT binding activity. This suggests that the 3rd TM, although it is essential, requires the other segment(s) located in the last half of human DAT for the CFT binding.

The same strategy was applied to determine the other segment(s) responsible for the CFT binding in the last half of human DAT. The last half of DAT can be divided into 3 segments encompassing the 6-8th TM, the 9-12th TM, and the carboxyl terminal tail using *Cla*I and *Pst*I restriction site at 1303, 1743, respectively. In human DAT, each of the 3 segments was replaced with the segment corresponding to bovine DAT to find out segment(s) responsible for the CFT binding. The substitution of the 9-12th segment didn't abolish but rather increased the CFT binding activity by 20% (see Chimera 8 in Table 3). However, the CFT binding activity was completely abolished by substituting the segment encompassing the 6-8th TM (in chimera 7), indicating the 6-8th TM is another structure required for the CFT binding. The substitution of the carboxyl terminal tail (in chimera 9) also decreased the CFT binding but it was not as drastic as that of the 6-8th TM.

Table 4. Both of the segments encompassing the 3rd and 6-8th TM are required for the CFT binding

		3rd TMD / 6-8th TMD	CFT binding (% hDAT)
hDAT		/	100.0±11.4
Chi 10		/	7.2±5.4
Chi 11		/	ND
Chi 12		/	86.1±17.3
Chi 13		/	151.5±29.6
Chi 14		/	104.0±13.2
Chi 15		/	ND
Chi 16		/	ND
Chi 17		/	152±10.6
Chi 18		/	ND
Chi 19		/	ND
Chi 20		/	ND
Chi 21		/	ND
Chi 22		/	ND
Chi 23		/	64.3±11.4
Chi 24		/	ND
Chi 25		/	ND

In the functional analyses of the subsequent chimeras, the CFT binding activities were exclusively detected in the chimeras in which both of the 3rd and the 6-8th TMD are composed of human DAT sequences. These findings strongly suggest that the CFT binding requires the human sequences at least in the regions encompassing the 3rd and the 6-8th TM. The values are means ± SEM of three to six independent experiments performed in duplicate. ND, not detected

The analysis of CFT binding activities in the chimeras 6, 7, 10, and 11 revealed that neither of the human

sequences in the 3rd or the 6-8th TM alone can elicit the CFT binding activity although they are important for the activity. To find out if both of the human segments encompassing the 3rd and the 6-8th TM are sufficient for the CFT binding, we construct the chimera 12 which contains human sequences only in these two segments and the bovine sequences in the rest of molecule. The CFT binding activity of chimera 12 (86.1 ± 17.3% of human DAT) is comparable with that of human DAT. Taken together, we conclude that the human sequences in the 3rd and the 6-8th TM are necessary and sufficient for the CFT binding to human DAT. The analysis of CFT binding activities for the subsequent chimeras (chimera 13-24) consolidate our conclusion. Without any exception, the CFT binding activities are avid in the chimeras of which the 3rd and 6-8th TM are consist of human sequences. In contrast, they are poorly detected if one or all of these segments is replaced with bovine one.

Possible role of the carboxyl terminal tail for the CFT binding

The CFT binding activity of human DAT decreased by 30% by substituting the carboxyl terminal tail with the equivalent bovine sequence (see chimera 9 in the table 3 and 6). Moreover, the additional substitution of human DAT carboxyl terminal sequence into chimera 11 in which the 3rd and the 6-8th TM are already consist of human sequences significantly increased the CFT binding by 70 %. Comparing the CFT binding activities between chimera 17 and 12 or 4 and 23, the CFT binding activities of chimera 17 and 4, of which carboxyl terminal tails are composed of human sequence, are significantly higher than those of 12 and 23, respectively (Table 5). These findings indicate that the carboxyl terminal tail enhances the CFT binding in DAT, even though it is not absolutely required.

Table 5. Carboxyl terminal tail of human DAT is not exclusively needed for CFT binding, but enhances the CFT binding

	CFT binding (% hDAT)
hDAT	100.0±11.4
Chi 9	70.2±10.0
Chi 17	152±10.6
Chi 12	86.1±17.3
Chi 4	73.3±13.0
Chi 23	64.3±11.4

Comparing the activities for the CFT binding between functional DAT proteins in which only carboxyl terminal tail are different (hDAT & chimera 9, chimera 17 & 12, and chimera 4 & 23), those activities are always higher in the DAT proteins that have human carboxyl terminal tail. These findings suggest that the carboxyl terminal tail is also required but not absolutely. The values are means ± SEM of three to six independent experiments performed in duplicate. ND, not detected

We have performed analyses of dopamine uptake and Parkisonian toxin MPP^+ uptake on the chimeric DATs. Although it is proven, through these anyalyses, that the 3rd TM and the carboxyl terminal tail is also important in the substrate transports (Lee *et al*., 1997, 1998) and that the 6-8th TM is possibly involved in the MPP^+ uptake, we couldn't find out more discrete manners for these substrate transports (Data not shown).

Discussion

Although both human and bovine DAT mediate the transport of dopamine into cells, CFT binding activities are highly discriminative between these two proteins. Because they are structurally similar, e.g. same topology and the overall 89% amino acid sequence identity, yet with distinct difference, chimeras between these two proteins are extremely informative for the structure-and-function relationship for the CFT binding. In the present studies, the functional analyses for the chimeric DATs of human and bovine reveal the structures responsible for the CFT binding and a cue for the mode of interaction of CFT to DAT.

We, previously, demonstrated that the 3rd TM of the human DAT, especially valine at the 152nd residue, is quite crucial for substrate transports and CFT binding (Lee *et al*., 1998; Lee *et al*., 2000). Likewise, it has also been suggested that the 3rd TM of the rat serotonin transporter contains residues associated with its functions (Chen *et al*., 1997). However, it is likely that the 3rd TM is not enough, but requires another domain(s) for the CFT binding based on the finding that only the substitution of the human 3rd TM cassette into bovine DAT did not produce the CFT binding activity. The present study shows that the regions, at least, encompassing the 3rd and the 6-8th TM should be composed of human DAT sequences to elicit CFT binding activities, and that the human sequence of carboxyl terminal tail enhance the CFT binding activities even though it is not absolutely required. If the chimeric DATs have human sequences of the 3rd and 6-8th TM, they showed conspicuous CFT binding activities without any exception. On the contrary, CFT binding activities were scarcely detected if either of two regions has bovine sequence. However, we couldn't find any discrete requirement of domains for substrate (dopamine and MPP^+) uptakes. Its probably because the substrate transport is mediated by a more complicated manner. These may include the binding of DA to the transporter, interactions of DA with amino acids during transport, the availability of transporter to Na^+, symport, membrane localization and so on.

In addition to the importance of the 3rd TM on the DAT functions, several studies have shown that the 6-8th TM is responsible for the affinities of psychostimulant drugs

such as cocaine and tricyclic antidepressants (Giros *et al.*, 1994; Roubert *et al.*, 2001). Recently, it has been suggested that the second loop outside the 3rd TM and the 7th TM have their spatial proximity in the tertiary structure through endogenous Zn^{2+}-binding sites even though they are separated in the primary structure and that physiologic concentration of Zn^{2+} markedly potentiates the cocaine binding (Norregaard *et al.*, 1998; Loland *et al.*, 1999). Our findings, combined with these previous studies, highly suggest that the regions encompassing the 3rd and the 6-8th TM might participate to form the pocket for CFT binding.

Several studies have presented that carboxyl terminal is important for the functions of several transporters (Lee *et al.*, 1996; Lee *et al.*, 1996a; Lee *et al.*, 1997; Cope *et al.*, 2000). Considering that the carboxyl tail is located in the cytoplasm, the role of carboxyl tail in CFT binding might be mediated by an indirect manner, such as by controlling membrane trafficking (Cope *et al.*, 2000) or protein expression of DAT.

Determination of the mode of interaction of CFT to DAT is prerequisite for the fundamental solutions of cocaine addict and intoxication. The present study focuses on elucidating the function-and-structure relationship for the CFT binding. It cannot yet be definitely inferred from our results the precise mechanism of the CFT binding. Further exploration of which residues in these domains are participated could help to resolve this issue.

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