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Human Genetics and Pharmacology of Neurotransmitter Transporters

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Abstract Biogenic amine neurotransmitters are released from nerve terminals and activate pre- and postsynaptic receptors. Released neurotransmitters are sequestered by transporters into presynaptic neurons, a major mode of their inactivation in the brain. Genetic studies of human biogenic amine transporter genes, including the dopamine transporter (hDAT; *SLC6A3*), the serotonin transporter (hSERT; *SLC6A4*), and the norepinephrine transporter (hNET; *SLC6A2*) have provided insight into how genomic variations in these transporter genes influence pharmacology and brain physiology. Genetic variants can influence transporter function by various mechanisms, including substrate affinities, transport velocity, transporter expression levels (density), extracellular membrane expression, trafficking and turnover, and neurotransmitter release. It is increasingly apparent that genetic variants of monoamine transporters also contribute to individual differences in behavior and neuropsychiatric disorders. This chapter summarizes current knowledge of transporters with a focus on genomic variations, expression variations, pharmacology of protein variants, and known association with human diseases.

Keywords Dopamine transporter · Serotonin transporter · Norepinephrine transporter · Single nucleotide polymorphisms · Transporter pharmacology

1 The Human Dopamine Transporter (*SLC6A3*)

1.1 Introduction

The neurotransmitter dopamine (DA) is implicated in a wide range of physiological processes (e.g., movement, cognition, memory, and reward) and pathophysiogical states. Deficits of brain DA are directly associated with motor impairment in Parkinson's disease and DA replacement therapy is the most effective treatment strategy for this disease. Conversely, D2 DA receptor antagonists are effective therapies for alleviating specific symptoms of schizophrenia, suggestive of excess DA neurotransmission in this psychiatric disorder. The human DA transporter (hDAT) is also a major target of therapeutic drugs and illicit drugs of abuse/addiction. DAT inhibitors (e.g., methylphenidate) or DAT substrates (e.g., amphetamine) effectively elevate extracellular DA levels and are widely used to treat attention deficit hyperactivity disorder (ADHD) and narcolepsy. A relatively weak DAT inhibitor bupropion is used therapeutically to treat depression in a subpopulation and to treat nicotine addiction in a wider cohort. Psychostimulant drugs of abuse, including cocaine, amphetamine, methamphetamine, and 3,4-methylene-dioxymethamphetamine (MDMA), target the DAT, albeit not exclusively (Madras et al. 1989; Verrico et al., 2005).

Cell bodies that produce DA are highly circumscribed in the substantia nigra, the ventral tegmental area and discrete regions of the hypothalamus, with terminals fanning out to the caudate nucleus, putamen nucleus accumbens, and frontal cortical regions. The DAT limits synaptic activity and diffusion mediated by DA, sequestering extracellular DA into neurons. The DAT is present on cell bodies, dendrites and axons, but apparently is not localized in the im-

mediate active zone of the synapse (Hersch et al. 1997; Nirenberg et al. 1996). Accordingly, the DAT diminishes DA overflow into perisynaptic regions, but may not affect DA levels within the synapse. The DAT is expressed in all DA neurons, with expression levels high in neurons originating in the substantia nigra and ventral tegmental area (Ciliax et al. 1995) and projecting to the striatum, nucleus accumbens, prefrontal cortex, and hypothalamus. Importantly, relative concentrations of DA, DAT, and DA receptors are consistent in the caudate-putamen, nucleus accumbens, and substantia nigra, with the DAT likely to regulate DA signaling strength and duration in these brain regions. The ratio of DAT to DA receptor expression levels is lower in other brain regions (De La Garza and Madras 2000), a mismatch that could result in DA clearance by metabolism, diffusion, or by another transporter. The frontal cortex expresses much lower levels of the DAT and DA autoreceptors, stores less DA, and relies more on DA synthesis than on vesicular recycling for DA release. Consequently, DAT-mediated DA regulation in the striatum cannot be liberally extrapolated to frontal cortex.

The critical role of the DAT in regulating DA neurotransmission and presynaptic homeostasis in the basal ganglia is clearly apparent in mice with null mutations of the DAT. DAT-mutant mice display robust phenotypic changes in behavior (hyperactivity, impaired care by females for their offspring), appearance (small size, skeletal abnormalities), physiological function (pituitary hypoplasia, sleep dysregulation), and brain function (cognitive and sensorimotor gating deficits), in comparison with non-mutant mice. Adaptations in the striatum of the mutant mice include a reduction in the DA-synthesizing enzyme tyrosine hydroxylase, vesicular DA stores, stimulated DA release, and D_1 and D_2 (but not D_3) DA receptor densities and function (review: Gainetdinov and Caron 2003).

At the molecular level, the 12-membrane-spanning DAT protein contains a large extracellular loop with consensus sites for glycosylation that function to regulate DAT trafficking and stability (Li et al. 2004). Potential phosphorylation sites (serine, threonine, and tyrosine) may also acutely modulate DAT trafficking and activity (review: Mortensen and Amara 2003). DA is released from both dendrites and axons and may activate receptors locally or remotely through volume transmission. DAT generates three types of ion channel-like conductances (Ingram et al. 2002; Sonders et al. 1997). In the substantia nigra, substrate transport by the DAT initiates an excitatory DAT-mediated current, cell depolarization, and consequent augmentation of somatodendritic DA release (Falkenburger et al. 2001; Ingram et al. 2002). This control of DA release is region-specific, as in the striatum; DA clearance is a primary function of the DAT.

1.1.1 DAT Regulation and Adaptation

Regulation of DAT density and function is relevant to physiological and pathophysiological processes in brain. Physiological turnover of the DAT protein in

rodent brain is approximately 2–3 days (Kimmel et al. 2000), but the DAT is also dynamically regulated. Ion gradients, phosphorylation activity, and other agents can modify DAT activity transiently or for extended periods, even after the agents are cleared from DAT (Gulley and Zahniser 2003; Mortensen and Amara 2003). Adaptive changes are reflected in density, activity, and cellular localization.

1.1.2 Transient Effects

The DAT can be regulated acutely by physiological, pharmacological, and activity-dependent mechanisms. Under normal conditions, the DAT is trafficked constitutively from the cell membrane to the intracellular milieu and then degraded or recycled to the cell surface (Daniels and Amara 1999; Loder and Melikian 2003). Activation of protein kinase C (PKC) pathways by phorbol esters reduces DA transport capacity and the number of binding sites on the cell surface (for reviews: Gulley and Zahniser 2003; Mortensen and Amara 2003). Acute exposure to substrates or inhibitors can also rapidly and reversibly modify DAT function. By promoting DAT trafficking (to the cell interior) and internalization, amphetamine acutely reduces DA transport capacity in rodent brain or cultured cells within 1 h of administration (Fleckenstein et al. 1999; Saunders et al. 2000). The DAT inhibitor cocaine, on the other hand, promotes upregulation of DAT density, function, DAT mobilization to the cell surface, and increased cell surface expression (Daws et al. 2002; Little et al. 2002). DA itself regulates the DAT, directly by downregulating surface expression, or indirectly by upregulating the DAT via D_2 DA autoreceptors (Gulley and Zahniser 2003). The DAT can act as a single unit, self-associate, or bind to other proteins. Oligomers of the DAT exist but again, their functional significance is not known (Sorkina et al. 2003; Torres et al. 2003).

1.1.3 DAT Regulation and the DAT Gene

DAT density may be altered by genetic abnormalities, but currently there are no candidate polymorphisms that correlate with DAT density. The 3′-untranslated region of the DAT gene has a fixed length repeat sequence that varies by number of repeats from 3–11 (Vandenbergh et al. 1992). Although the 10-repeat sequence is the most common form of the gene, inheritance of this repeat length in both alleles is associated with ADHD in some studies, but accounts for less than 4% of the variance (Cook et al. 1995; Madras et al. 2002; Waldman et al. 1998). The repeat number in this region of the gene and association with DAT density is controversial. One study demonstrated a higher DAT density with the 10/10 repeat genotype (Heinz et al. 2000); another study found a lower density in the 10/10 than the 9/10 repeat (Jacobsen et al. 2000);

a third study found no difference in DAT density (Martinez et al. 2001); and a fourth study found a higher DAT in the 9/9 repeat (van Dyck et al. 2005).

1.2 DAT Genomic Variants

1.2.1 Introduction

The large hDAT gene (51.7 kb) displays a high density of polymorphisms (>7/kb). It is estimated that more than 400 single nucleotide polymorphisms (SNPs) are located in this gene (51.7 kb from exons 1 to 15). More than 100 SNPs located in the 5′ region (16 kb from the upstream gene *FLJ12443* to exon 1) have been deposited into the National Center for Biotechnology Information (NCBI) dbSNP (Build 124). Less than 50 of the polymorphisms, including approximately 19 SNPs and one variable number of tandem repeat (VNTR) in the exons, have been documented in the literature (Greenwood et al. 2001; Greenwood and Kelsoe 2003; Grunhage et al. 2000; Vandenbergh et al. 1992, 2000) and most of them have been included in the dbSNP. This information suggests that *SLC6A3* is subject to extensive variations among individuals, and some of these variations perhaps confer risk factors for *SLC6A3*-related diseases. Listed in Fig. 1A are polymorphisms that have been used either in association studies and analyzed for effects on gene expression or cause missense mutations (indicated in the parentheses) in the hDAT protein. rs2975226, located immediately upstream of exon 1, is a T/A SNP and has been used in two association studies (see Sect. 1.5).

1.2.2 Coding Region Variants

The coding region of DAT is highly conserved. Re-sequencing of 551 Caucasian individuals for the coding region revealed 15 exon SNPs including 4 missense mutations, none of which have frequency exceeding 1% (Table 1; Grunhage et al. 2000; Vandenbergh et al. 2002). This information suggests that wildtype function of hDAT is required for human survival.

1.2.3 Non-coding Region Variants

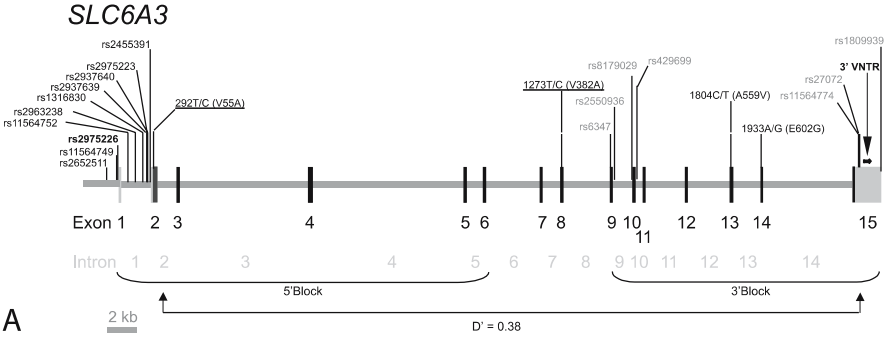
The 3′ VNTR (40 bp repeats with 92% identity located from 2741 to 3144 in exon 15) contains at least 7–11 repeats of 40 bp, but the 9- and 10-repeats are the most frequent alleles (Doucette-Stamm et al. 1995; Nakatome et al. 1995). This 3′ VNTR has been the most popular genetic marker of this gene for more than 100 association studies during the last decade. Besides the 3′ VNTR, there are two additional islands of imperfect tandem repeats downstream. One is

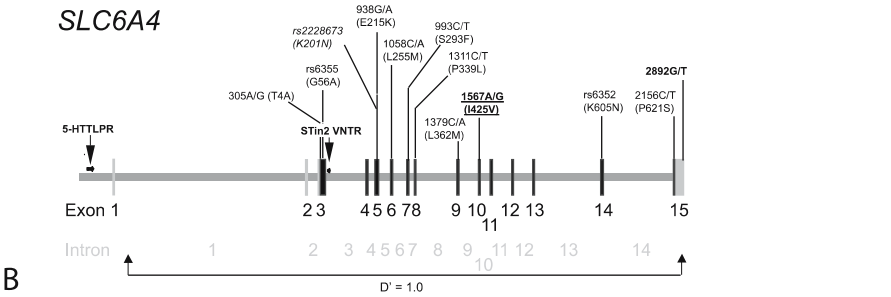
2.6 repeats of 82 bp (90% identity located 475 bp downstream of the 3′ VNTR or from 3619 to 3830) and designated as 3′ TR2 here. Another is 5 repeats of 38 bp (75% identity located 486 bp downstream of 3′ VNTR or from 3630 to 3830) and designated 3′ TR3 here. 3′ TR2 and 3′ TR3 overlap each other for most of the regions. There is no information on whether 3′ TR2 and 3′ TR3 are variable.

Linkage disequilibrium (LD) between 5′ and 3′-ends of *SLC6A3* is low. The 5′ SNP rs11564749 (P+215 in Greenwood et al. 2001) displays D′ of less than 0.3 when pairing with eight different SNPs from exons 9 and 15, including the seven synonymous SNPs listed in Fig. 1A, based on analysis of hundreds of Caucasian chromosomes of northern European descent (Greenwood et al. 2002). There are two blocks of high LD, from exon 1 to intron 6 (5′ block) and from exons 9 to 15 (3′ block, see Fig. 1A; Greenwood et al. 2002). The HapMap database (http://www.hapmap.org/index.html.en, Release 16c.1, 15 June 2005) shows that LD between rs2617605 in intron 2 (not shown in Fig. 1A)

Fig. 1 A–C Variations of the human DAT (**A**, *SLC6A3*), serotonin transporter (SERT) (**B**, *SLC6A4*), and norepinephrine transporter (NET) (**C**, *SLC6A2*) genes, which are located on chromosomes 5p15.3, 17q11.1, and 16q12.2, respectively. *Horizontal gray lines* are chromosomal regions harboring the genes; the *crossing vertical bars* are exons (*black* for translated and *gray* for untranslated regions), and between exons are introns as *numbered underneath*. *Vertical lines* indicate the locations of SNPs, and wherever possible, dbSNPs (*rs*) are used; *vertical arrows* indicated genetic markers (*horizontal arrows*) of simple sequence repeats such as VNTR (*SLC6A3*, STin2 VNTR of *SLC6A4*) and indel (5-HTTLPR of *SLC6A4* and NETpPR of *SLC6A2*). For those that are not rs, coordinates of mRNAs (NM_001044 an entry on 2 May 2005 for *SLC6A3*, NM_001045 entered on 10 June 2005 for *SLC6A4* and AF061198 as reported in Kim et al. 1999 for *SLC6A2*) are used. *Parentheses* indicate amino acid variations caused by non-synonymous SNPs. *Bold* labels indicate markers (or haplotypes in *gray* of panel *a*) that displayed positive association with human diseases and *underlined* are variants that have been analyzed for transporter activity. *Italics* indicate these listed in the dbSNP database (Build 124) but not in the literature. Note for *SLC6A3*: two blocks of high LD are indicated as 5′ and 3′ blocks, based on LD analysis (Greenwood et al. 2002). Notes for *SLC6A2*: [a]originally reported as T-182C in (Zill et al. 2002); [b]originally reported as 1287G/A in (Stober et al. 1996); [c]originally reported as an intron 8 SNP by (Ono et al. 2003), but re-designated here because of the discovery of new exon 1 (Kim et al. 1999); three indicated blocks are haplotypes conserved in different populations, and block 1 may extends to 10.5 kb downstream of exon 15 (Belfer et al. 2004). Variation information came from the following references: Greenwood et al. (2001), Greenwood and Kelsoe (2003), Grunhage et al. (2000), and Vandenbergh et al. (2000) for *SLC6A3*; Battersby et al. (1996 and 1999), Delbruck et al. (1997 and 2001), Di Bella et al. (1996), Heils et al. (1996), Ogilvie et al. (1996), and Ozaki et al. (2003) for *SLC6A4*; and Hahn et al. (2005), Halushka et al. (1999), Iwasa et al. (2001), Ono et al. (2003), Shannon et al. (2000), Stober et al. (1996 and 1999), and Zill et al. (2002) for *SLC6A2*; and the NCBI databases (dbSNP Build 124 and MapView Build 35.1). At the *bottom* of each panel is the D′ value for LD in American Caucasians, based on HapMap (http://www.hapmap.org/index.html.en). See text for details on the SNPs used to calculate the D′ values. *Scale bar*: 2 kb

and rs27072 in exon 15 display D′ = 0.375 for 59 Caucasians (CEPH, Utah residents with ancestry from North and Western Europe), 0.436 for 45 Chinese (Han Chinese Beijing), 0.18 for 43 Japanese (Tokyo), and 0.092 for 60 Africans (Yoruba Ibaden, Nigeria). The 3′ block is diminished to some extent in the non-Caucasian populations. These data are consistent with the idea that there is very low LD between the 5′ promoter region and 3′ VNTR and that DAT gene variations differ significantly among different populations.





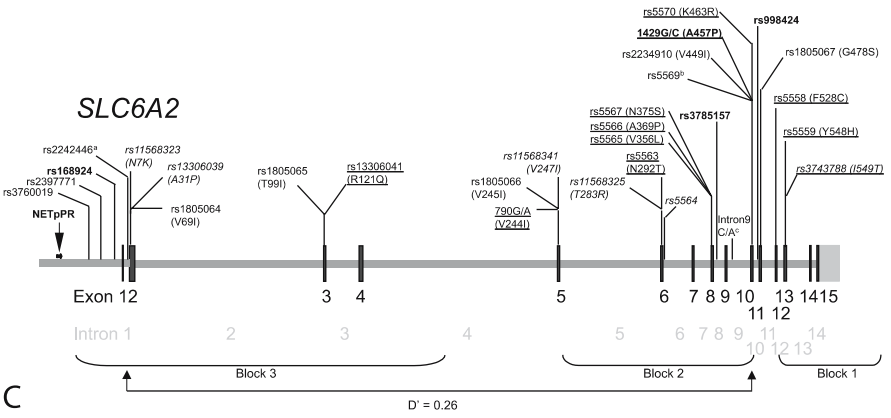


Table 1 Frequency (%) of human neurotransmitter transporter variants

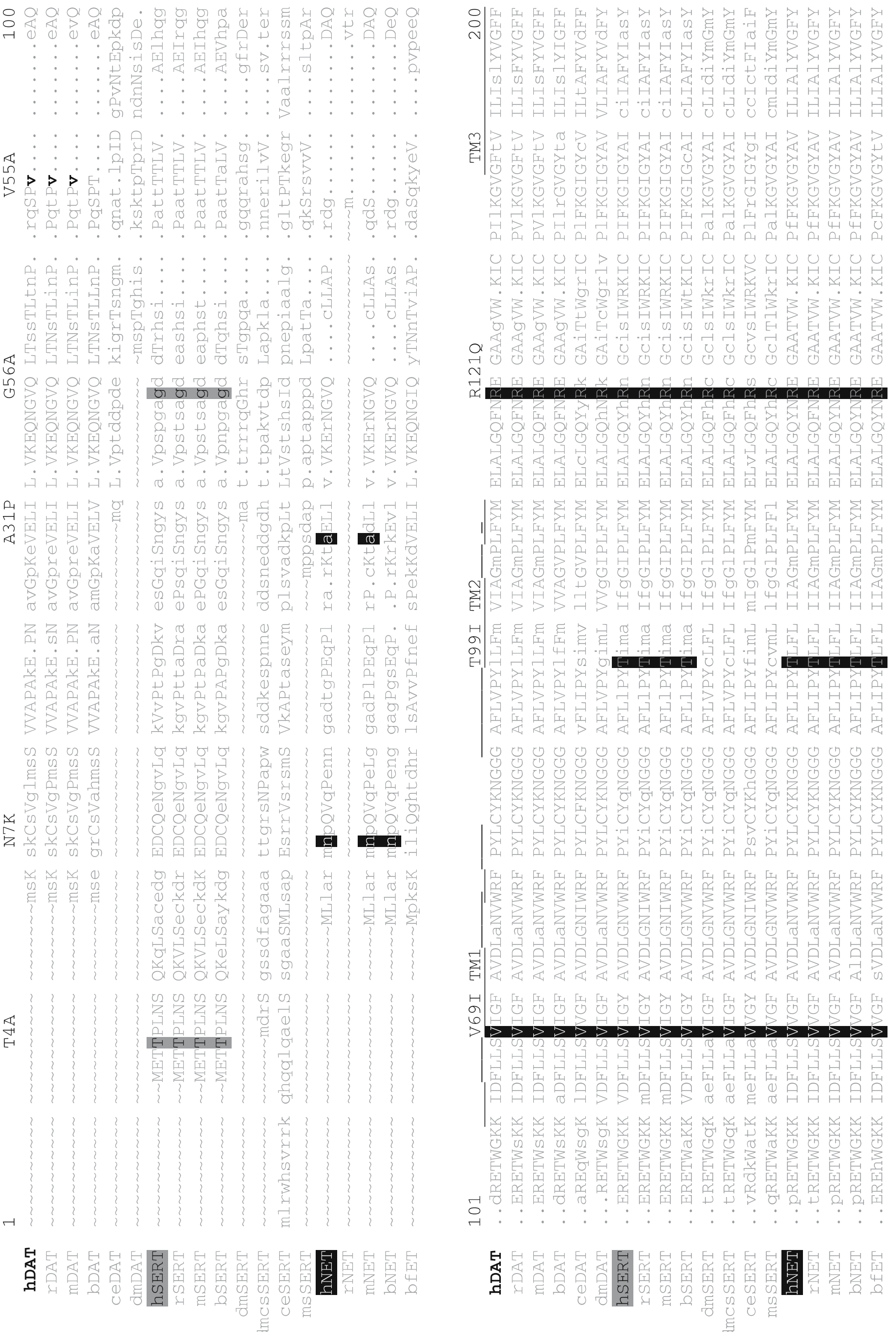
hDAT	hSERT		hNET	
V55A 1%[a]	T4A 0.1%[c]	P621S 0.1%[c]	N7 K 0.2%[d]	V356L 4.3%[f]
V382A* 1%[a]	G56A 1.7%[d]		A31P ?	A369P** 2.3%[f]
A559V 0.5%[b]	K201N 15.7%[d]		V69I 0.3%[i]	N375S 4.3%[f]
E602G 0.5%[b]	E215K 0.1%[c]		T99I 1.8%[i]	V449I 0.07%[i]
	L255M 0.1%[h]		R121Q* 0.5%[e]	A457P** ∼1%[g]
	S293F 0.1%[c]		V244I ?	K463R 10.0%[d]
	P339L 0.1%[c]		V245I 0.43%[i]	G478S 0.07%[i]
	L362M 0.1%[c]		V247I 0.2%[d]	F528C 5.5%[d]
	I425V#0.1%[c]		T283R 0.4%[d]	Y548H 4.7%[d]
	K605N 0.1%[c]		N292T* 4.3%[d]	I549T 1.4%[d]

*Uptake activity reduced by 37%–50%, **Uptake activity reduced by approximately 99% (Hahn et al. 2003, 2005; Lin and Uhl 2003), #Gain-of-function (Kilic et al. 2003; Runkel et al. 2000); see text for details, [a]Vandenbergh et al. 2000, [b]Grunhage et al. 2000, [c]Glatt et al. 2001, [d]dbSNP, [e]Iwasa et al. 2001, [f]Halushka et al. 1999 and dbSNP (Build 124), [g]Shannon et al. 2000, [h]Di Bella et al. 1996, [i]Runkel et al. 2000

1.3 DAT Protein Variants

To date, only 4 hDAT protein variants have been reported, including V55A, V382A, A559V, and E602G. The NCBI dbSNP database (Build 124) has not shown any additional protein variants yet. V55A and V382A have been stud-

Fig. 2 Conservation of variable amino acid residues of hDAT, hSERT, and hNET as demonstrated by alignment of 19 transporter amino acid sequences. *Black* and *gray* highlight the conservation of human transporter residues: *black* and *bold* with *clear background* for hDAT, *black with gray background* for hSERT, and *white with black background* for hNET; the corresponding variations are indicated on *top* of alignment. Transmembrane domains (*TM*s) are covered by *horizontal lines*. Prefix for transporter names: *h*, human; *r*, rat; *m*, mouse; *b*, bovine; *bf*, bullfrog (*Rana catesbeiana*); *dm*, *Drosophila melanogaster*; *dmcs*, *Drosophila melanogaster* Canton S; *ce*, *Caenorhabditis elegans*; *ms*, *Manduca sexta*. *DAT*, dopamine transporter; *SERT*, serotonin transporter; *NET*, norepinephrine transporter; *ET*, L-epinephrine transporter. Sequence sources as GenBank accession No.: hDAT, L24178; rDAT, M80570; mDAT, AF109072; bDAT, M80234; hNET, M65105; rNET, AB021971; mNET, U70306; bNET, U09198; fET, U72877; hSERT, L05568; rSERT, M79450; msSERT, AAN59781; bSERT, AF119122; dmSERT, U02296; dmcsSERT, U04809; ceDAT, AF079899; dmDAT, AAF76882; ceSERT, AAK84832; and msSERT, AAN59781. The last few C-terminal residues are irrelevant and not shown here



hDAT
rDAT
mDAT
bDAT
ceDAT
dmDAT
hSERT
rSERT
mSERT
bSERT
dmSERT
dmcsSERT
ceSERT
msSERT
hNET
rNET
mNET
bNET
bfET
1
T4A
N7K
A31P
G56A
V55A
100
101
V69I
TM1
T99I
TM2
R121Q
TM3
200

Fig. 2 (continued)

Fig. 2 (continued)

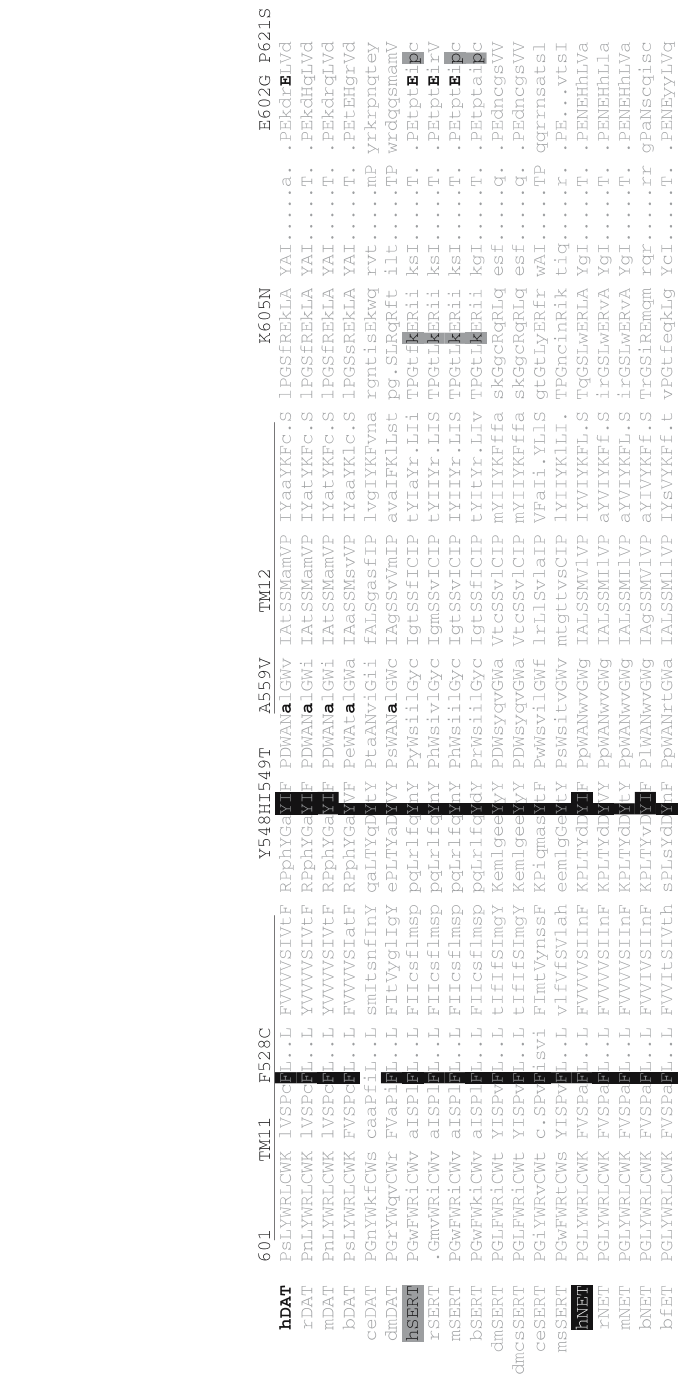


Fig. 2 (continued)

ied for pharmacological activities. The N-terminally located V55 is conserved in DATs from human, rat, and mouse but not from bovine, *Caenorhabditis elegans*, or *Drosophila*. This V55 is not present in any serotonin transporters (SERTs) or norepinephrine transporters (NETs) either (Fig. 2). Such modest conservation indicates lack of essentiality of V55 in DAT function. Consistently, functional analysis showed that V55A displays normal in vitro expression and pharmacological profiles in regards to DA uptake V_{max}, affinity for DA, cocaine, mazindol, and *d*-amphetamine in inhibition of [^{3}H]DA uptake experiments; the exception was that the DA uptake affinity decreased by 1.7-fold (Lin and Uhl 2003). By contrast, the extracellular loop 4 (ECL4)-located V382 is conserved in all of DATs, SERTs, and NETs cloned from seven different species so far, suggesting that V382 plays an important role in transport (Fig. 2). In vitro expression assays showed that Ala substitution for V382 disrupts plasma membrane targeting of DAT in COS-7 cells so that the DA uptake V_{max} and cocaine analog [^{3}H]2-β-carbomethoxy-3-β-(4-fluorophenyl) tropane (CFT) binding B_{max} values are reduced by 50% (uptake and binding affinities remain normal). Importantly, this disrupted expression is dominant to the wildtype protein expression. Besides the influence on plasma membrane expression levels, mutation V382A decreases affinity by 6.3-fold for DA in inhibiting [^{3}H]CFT binding but increases affinity by 1.3- to 1.6-fold for cocaine, benztropine, and GBR12909 in inhibition of [^{3}H]DA uptake experiments (Lin and Uhl, 2003; Lin et al. 1999).

A559V and E602G have not been examined yet. A559 is located at the boundary between extracellular space and transmembrane segment (TM)12 and is present in hDAT, rat (r)DAT, mouse (m)DAT and *Drosophila melanogaster* (dm)DAT but not in *Caenorhabditis elegans* (ce)DAT or any of the SERTs and NETs. At this position, a Val residue is present in ceDAT and rSERT. The C-terminally located E602 is present in only the hDAT out of the six DATs listed in Fig. 2. At this position, Gly is present in bovine (b)DAT and the dmSERTs. It is unlikely that E602G displays disrupted expression. Overall, the residues at these two positions are conserved for none of the SERTs and NETs either. It is reasonable to expect normal activity of both A559V and E602G.

1.4 Variations in DAT Expression Levels

hDAT expression levels differ significantly from individual to individual. Postmortem studies have shown that the differences in [^{3}H]WIN 35428 binding to hDAT could be as large as fourfold among different individuals, suggesting that hDAT expression differs significantly from individual to individual (Little et al. 1999). The question becomes whether *SLC6A3* markers are associated with such differences. It has been an interesting subject for a long time as to whether the 3′ VNTR, the most popular *SLC6A3* genetic marker, is correlated with hDAT expression levels in human brain. In vitro studies

have shown that SV40 promoter-driven luciferase gene (luc^+) tagged with the 9-repeat allele at 3′-end expressed Luc activity 33% higher than that tagged with the 10-repeat allele in vitro (Miller and Madras 2002). The correlation between 3′ VNTR and hDAT availability in human striatum using single-photon emission computed tomography (SPECT) imaging technology has been investigated. In 30 healthy subjects, individuals carrying the 9/10 repeat alleles displayed higher DAT availability than those carrying the 10/10 repeat alleles, which reached statistical significance ($F = 9.43$, $df = 1, 24$, $p = 0.005$) (Jacobsen et al. 2000). Five years later, they reported data from 96 healthy subjects suggesting that 9 repeat carriers ($n = 41$, 49.8 +/− 19.5 years) had a mean striatal DAT availability 8.9% higher than the 10 repeat homozygotes ($n = 53$, 49.9 +/− 19.2 years), which again reached statistical significance [$F(1.93) = 6.25$, $p = 0.014$] (van Dyck et al. 2005). These well-designed in vitro and in vivo studies have clearly shown the 10-repeat allele is associated with downregulated hDAT expression. Studies of smaller sample sizes in different populations have shown different results, reflecting the fact that hDAT availability in the human striatum varies significantly from individual to individual (Contin et al. 2004; Heinz et al. 2000; Martinez et al. 2001; Mill et al. 2002). These studies show clearly that hDAT availability declines significantly during aging. However, there is no evidence for individual differences in the declining rate.

Haplotypes conferred by the nine 5′ SNPs (upstream of exon 2, see Fig. 1A) have been analyzed for promoter activity in vitro. A luciferase reporter construct containing haplotype TTTAAAGAC displayed promoter activity 1.4-fold higher than one containing CAGCGGAGT. Including the haplotypes conferred by the selected seven 3′ SNPs (from intron 8 to exon 15, Fig. 1A) into the 5′ reporter constructs increased promoter activity by approximately twofold (Greenwood and Kelsoe 2003).

1.5 DAT Association with Human Diseases

The DAT gene is associated with more than ten human diseases. The majority of clinical genotyping of hDAT is focused on ADHD, primarily because ADHD has a high genetic load and the DAT is a principal target of anti-hyperactivity medications. Indeed, among the 16 association studies using 3′ VNTR as a DAT marker, 10 studies have demonstrated a positive association with ADHD, in Caucasians of American, Irish, British, and Canadian nationality and in Chinese populations (Table 2). A recent meta-analysis summarized genetic studies spanning 14 years (1991–2004) and found a positive association between the DAT gene and ADHD (Bobb et al. 2005b). A more recent SPECT study showed that reduced DAT availability in midbrain is found in ADHD patients, continuing to support this positive association (Jucaite et al. 2005). In vitro, in vivo imaging, and genetic studies, including DAT knockdown an-

Table 2 *SLC6A3* association with human diseases

Marker	Diseases	Association (No. of studies)		Reference(s)
3′ VNTR	ADHD	Yes	(10)	A
		No	(6)	B
		Yes by meta-analysis		C
	Poor response to	Yes	(3)	D
	Ritalin treatment	No	(0)	
	Alcoholism	Yes	(10)	E
		No	(2)	F
	Illegal drug abuse	Yes	(3)	G
		no	(1)	H
	Smoking	Yes	(6)	I
		No	(1)	J
3′ Haplotypes[a]	Bipolar disorder	Yes	(1)	K
		No	(0)	
5′ SNP rs2975226	Schizophrenia	Yes	(1)	L
		No	(0)	
	Bipolar disorder	Yes	(1)	M
		No	(0)	

[a]Inferred from the seven SNPs (in gray) located from intron 8 to exon 15 in Fig. 1a, [b]Using rs27072 which is close to 3′ VNTR (Fig. 1a), A: Barr et al. 2001; Chen et al. 2003; Cook et al. 1995; Gill et al. 1997; Maher et al. 2002; Oh et al. 2003; Payton et al. 2001b; Qian et al. 2003; Rowe et al. 2001; Waldman et al. 1998, B: Curran et al. 2001; Holmes et al. 2000; Muglia et al. 2002; Payton et al. 2001a; Roman et al. 2001; Todd et al. 2001, C: Bobb et al. 2005b, D: Kirley et al. 2003; Ling et al. 2004; Roman et al. 2002; Winsberg and Comings 1999, E: Dobashi et al. 1997; Galeeva et al. 2001; Gorwood et al. 2003; Limosin et al. 2004; Muramatsu and Higuchi 1995; Sander et al. 1997; Schmidt et al. 1998; Ueno et al. 1999; Wernicke et al. 2002, F: Chen et al. 2001; Franke et al. 1999, G: Blum et al. 1997; Gelernter et al. 1994; Ujike et al. 2003, H: Hong et al. 2003, I: Audrain-McGovern et al. 2004; Erblich et al. 2004; Lerman et al. 1999; Ling et al. 2004; Vandenbergh et al. 2002, J: Jorm et al. 2000, K: Greenwood et al. 2001, L: Khodayari et al. 2004, M: Keikhaee et al. 2005

imal studies, have almost reached a consensus that hDAT expression levels in the midbrain/striatum may confer a risk factor for ADHD (Fischman and Madras 2005; Madras et al. 2005; Miller and Madras 2002; Zhuang et al. 2001). Furthermore, the 3′ VNTR 10-repeat carriers, who have lower DAT expression levels and high risk for ADHD, are prone to be resistant to methylphenidate treatments as shown by three studies in U.S., Brazil, and Ireland (Kirley et al. 2003; Roman et al. 2002; Winsberg and Comings 1999). In addition, the DAT gene appears to be associated with Tourette's syndrome in U.S. and German Caucasians (Comings et al. 1996; Muller-Vahl et al. 2000; Rowe et al. 1998).

The DAT has also been implicated in behavioral components of alcohol, cigarette, cocaine, and methamphetamine abuse (Table 2). The frequencies of positively associated 3′ VNTR 10-, 9-, and 7-repeat alleles are 5:8:2 (Blum et al. 1997; Dobashi et al. 1997; Erblich et al. 2004; Galeeva et al. 2001; Gelernter et al. 1994; Gorwood et al. 2003; Lerman et al. 2003; Muramatsu and Higuchi 1995; Sander et al. 1997; Schmidt et al. 1998; Ueno et al. 1999; Ujike et al. 2003; Vandenbergh et al. 2002; Wernicke et al. 2002). These data suggest that the 9-repeat allele is more involved than the 10-repeat allele in these behavioral paradigms, which is different from the findings from the ADHD studies in which the 10-repeat allele is implicated. Substance abusers are frequently comorbid for neuropsychiatric disorders. Although complex and polygenetic in nature, it is of considerable interest to determine overlap between candidate genes associated with substance abuse and neuropsychiatric disorders.

Many studies using single markers, including the 3′ VNTR near the 3′-end of the DAT gene, have shown negative associations with several diseases. Of approximately ten association studies on bipolar disorder and the 3′-VNTR or SNPs located at the 3′-end of the DAT gene, none was indicative of an association. However, in a study using haplotypes conferred by SNPs located from exon 9 to 15 (Fig. 1A), significant association with bipolar disorder was discovered (Greenwood et al. 2001).

Sequence analysis of the coding region of the DAT gene from 551 Caucasian individuals revealed 15 exon SNPs including 4 missense mutations (Grunhage et al. 2000; Vandenbergh et al. 2000). None of these SNPs displayed any association with alcoholism, Tourette's syndrome, or bipolar disorder in these samples. The four missense mutations are rare, all with a frequency of less than 1% and none of them has been implicated in any disease yet. Nevertheless, it is still possible that other polymorphisms of low LD with the 3′-VNTR or these SNPs regulate DAT activity. Based on long-standing views on dopaminergic involvements in pathogenesis of schizophrenia and Parkinson's disease, more than 20 association studies have been done using the 3′ VNTR and the majority of them showed no association with these diseases.

Interestingly, two recent studies that used rs2975226 (T/A) located immediately upstream of exon 1 showed a strong association of the T allele with both bipolar disorder and schizophrenia (Keikhaee et al. 2005; Khodayari et al. 2004). These results suggest two things. First, since this T allele is located on a haplotype that displayed higher promoter activity in vitro, high promoter activity in certain brain regions might confer a risk factor for bipolar disorder and schizophrenia if these promoter marker-based findings can be validated in different studies. Second, markers in the 5′ promoter region should be used in future studies not only to clarify the inconsistencies of the current 3′ VNTR-based association findings, but also to investigate DAT as a risk factor for other DA-related diseases.

2
The Human Serotonin Transporter (*SLC6A4*)

2.1
Introduction

Serotonin contributes to and modulates a wide range of functions in the periphery, including vasoconstriction, gastrointestinal motility, and secretion. In normal brain states, serotonin is implicated in mood, sleep, appetite, and in the production of anxiety, fear, reward, and aggression. Dysfunction of brain serotonin signaling is implicated in several neuropsychiatric disorders, including depression, suicide, obsessive-compulsive disorder (OCD), migraine headaches, eating disorders, and autism. Neurons that produce and release serotonin are few in number, highly circumscribed in discrete raphe nuclei in the brainstem, and project to multiple brain regions (cortex, thalamus, basal ganglia, hippocampus, amygdala, and others), as well as the medulla and spinal cord (review: Jacobs and Azmitia 1992).

Serotonin activates more than 15 serotonin receptor subtypes that display unique distribution, structure, pharmacology, and signaling pathways. Implicated in mediating the wide array of physiological effects of serotonin, the activity of these receptors is regulated by SERT, which determines serotonin availability in brain. Encoded by a single gene, variations in SERT activity and density and SERT polymorphisms are implicated in depression, anxiety, OCD, suicide, autism, and substance abuse (Bastani et al. 1991; Malison et al. 1998; Newport et al. 2004; Owens and Nemeroff 1998; Paul et al. 1981). Not surprisingly, the SERT is a principal target of a range of therapeutic and illicit drugs of abuse. SERT activity is blocked by tricyclic and serotonin-selective reuptake inhibitors (SSRIs), drugs widely used in the treatment of depression, OCD, and other psychiatric diseases. Cocaine and amphetamines block SERT function, a process implicated in the psychoactive and addictive properties of the drugs (Eshleman et al. 1997; Verrico et al. 2005).

SERT can be regulated acutely by receptors, signal transducers, and kinase activity which rapidly modulate SERT expression (Anderson and Horne 1992; Miller and Hoffman 1994; Myers and Pitt 1988). In common with the DAT and NET, PKC activation leads to a rapid loss of surface-expressed SERT (Apparsundaram et al. 1998; Blakely et al. 1998; Miranda et al. 2005; Qian et al. 1997). In contrast to the DAT or NET, however, SERT substrates promote retention of the SERT on the membrane surface, whereas substrates for the DAT and NET might promote transporter internalization (Mao et al. 2004; Saunders et al. 2000).

The amino acid composition of the SERT is approximately 50% homologous to the DAT and NET, and its protein structure models similarly to the DAT and NET, with 12 transmembrane hydrophobic domains and intra- and extracellular loops of varying lengths. SERT messenger RNA (mRNA) expression is restricted to serotonin cell bodies in the raphe nuclei, whereas SERT protein is

widely distributed in brain, following the pattern of extensive innervation of cortical and subcortical regions of brain, as noted above.

2.2 SERT Genomic Variants

2.2.1 Introduction

The SERT (or 5-HTT) genomic gene sequence is smaller (37.8 kb) than the DAT but highly polymorphic. More than 100 SNPs have been identified in this gene (from exons 1 to 15) and more than 35 SNPs were found in the 5′ region (12.6 kb from the upstream bleomycin hydrolase gene to exon 1) and deposited in dbSNP (Build 124). Less than 20 polymorphisms located in the 5′ promoter region, intron 2, and exons have been documented in the literature, with the majority also documented in dbSNP (Fig. 1B).

2.2.2 Coding Region Variants

Overall, the coding region of the SERT is highly conserved. From re-sequencing of more than 450 individuals, only 11 missense mutants have been reported and nine of them have a very low frequency (0.1%, Table 1, but see following section). This information suggests that wildtype function of SERT is required for human survival.

2.2.3 Non-coding Variants

The SERT-linked polymorphic region (5-HTTLPR or SERTLPR), located at approximately −1.4 kb upstream of exon 1, has 14 (*s*), 15, 16 (*l*), 19, 20 (*xl*), 22-repeats of 20–23 bp and both the 14- and 16-repeat alleles have minor variations (Delbruck et al. 1997; Heils et al. 1996; Nakamura et al. 2000). This 5-HTTLPR of the 5-HTT has been used as a marker in hundreds of association studies. Between 5-HTTLPR and exon 1 is a 381-bp indel, but there is little information available on this indel (Flattem and Blakely 2000). The second-most-used marker is the intron 2 VNTR (also termed STin2, Fig. 1B). VNTR contains 9, 10, and 12 repeats of 17 bp (STin2.9, STin2.10 and STin2.12) (Ogilvie et al. 1996).

The allelic frequency for 5-HTTLPR and VNTR varies significantly from population to population (Table 3). The *s* allele is associated with alcoholism, anxiety, suicide, and depression. The lowest frequency of the *s* allele is found in African populations and highest frequency in Chinese and Japanese cohorts. Nonetheless, the association of the *s*-allele with these disorders in the various populations has not been systematically investigated. In contrast, the *l* allele has the highest frequency in African populations. It would be interesting to

Table 3 Allele frequency of 5-HTTLPR and VNTR in different populations

5-HTTLPR[a]h		American	European[b]	East Asian[c]	African[d]
	Allele				
	l	57%–69%	54%–61%	10%–37%	79%–84%
	s	29%–44%	39%–46%	63%–79%	9%–21%
	xl	2%	0%	0%–1.6%	6%

VNTR[e]		European-American	African-American	Japanese
	STin2.9	1.1%	1%	0%
	STin2.10	46.6%	26.0%	2.3%
	STin2.12	52.3%	73.1%	97.7%

[a]Delbruck et al. 2001; Nakamura et al. 2000; Ogilvie et al. 1996, [b]French and German, [c]Chinese and Japanese, [d]Central African, [e]Gelernter et al. 1997

determine whether Africans have higher frequency of *l*-associated diseases such as pulmonary hypertension and ADHD (see Sect. 2.5). The 15-, 19-, and 22-repeat alleles of 5-HTTLPR are reported to have frequency of less than 0.8% in Japanese, but not in Caucasians (Nakamura et al. 2000). For the VNTR, STin2.10 and STin.12 are the major alleles in European Americans and African Americans, but in Japanese populations STin.12 dominates, with a frequency of 97.7% (Gelernter et al. 1997). The sequencing information indicates that the SERT is considerably variable in individuals and among different populations. Besides the 5-HTTLPR and VNTR regions, an SNP, 2892G/T, located the 3′-end of exon 15 has been used in association studies (Table 4). Eleven other

Table 4 *SLC6A4* association with human diseases

Marker/disease	Association (No. of studies)[a]		Reference(s)
5-HTTLPR			
Trait anxiety	Yes	(Meta of 26)	Schinka et al. 2004
Major depression	Yes	(2 meta-analyses)	Furlong et al. 1998; Lotrich and Pollock 2004
	No	(Meta of 15)	Lasky-Su et al. 2005
Alcohol dependence	Yes	(18)	Feinn et al. 2005; Gorwood et al. 2004; Hu et al. 2005
Suicide behavior	Yes	(Meta of >18)	Anguelova et al. 2003; Lin and Tsai 2004
Aggression	Yes	(7)	Cadoret et al. 2003; Courtet et al. 2001; Han et al. 2004; Klauck et al. 1997; Retz et al. 2004; Zalsman et al. 2001

Table 4 (continued)

Marker/disease	Association (No. of studies)[a]		Reference(s)
	No	(2)	Baca-Garcia et al. 2004; Patkar et al. 2002
TCI/TPQ	Yes	(Meta of 16)	Munafo et al. 2005
Harm avoidance	No	(Meta of 8)	Sen et al. 2004
NEO neuroticism	Yes	(Meta of 10)	Sen et al. 2004
	No	(Meta of 13)	Munafo et al. 2005
Bipolar disorder	Yes	(4 meta-analyses)	Furlong et al. 1998; Lasky-Su et al. 2005; Levinson 2005; Lotrich and Pollock 2004
	No	(Meta of 43)	Cho et al. 2005
Autism	Yes	(2)	Cook et al. 1997; Klauck et al. 1997
Anorexia nervosa	Yes	(2)	Fumeron et al. 2001; Matsushita et al. 2004
	No	(1)	Sundaramurthy et al. 2000
Smoking (s)	Yes	(1)	Gerra et al. 2005
Alzheimer's disease	Yes	(1)	Li et al. 1997
dPIBS[c] in women	Yes	(1)	Cook et al. 1995
Pulmonary hypertension Intron 2 VNTR	Yes	(1)	Eddahibi et al. 2003
Schizophrenia	Yes	(Meta of 12)	Fan and Sklar 2005
Aggression 5′ haplotypes[b]	Yes	(1)	Davidge et al. 2004
Autism Haplotype l ± STin2.12	Yes	(1)	Conroy et al. 2004
Smoking Exon 9 rs6353 (I425 V)	Yes	(1)	Kremer et al. 2005
OCD 3′ SNP 2892G/T	Yes	(1)	Ozaki et al. 2003
Unipolar disorder 5-HTT haplotypes	Suggestive	(1)	102 ($p = 0.034$)
ADHD	Yes	(Meta of > 8)	Bobb et al. 2005b; Curran et al. 2005

[a]Meta as meta-analysis; no single method is sufficient for assessing evidence of publication bias (Furlong et al. 1998) and this is why multiple meta-analyses are listed here
[b]Inferred from 5-HTTLPR, SNPs, and STin2 (VNTR)
[c]Diarrhea-predominant irritable bowel syndrome

polymorphisms listed in Fig. 1B cause missense mutations (indicated in the parentheses) in the SERT protein.

Based on information available in the HapMap database, the hSERT gene displays high LD for most populations. The D′ value [for the region between rs2020933 located at 0.95 kb after exon 1 and rs1042173 located 0.1 kb to the 3′-end of exon 15 that covers almost the entire transcribed region (Fig. 1B)], is 1.0 for Caucasians (n = 56), Japanese (n = 44) and Africans (n = 60), but is 0.128 for Chinese (n = 45). The D′ value between rs2066713 located 1.7 kb before exon 2 and rs1042173 in exon 15 is 0.78 for Caucasians, 0.70 for Chinese, 1.0 for Japanese, and 0.609 for Africans.

2.3 SERT Protein Variants

Ten human SERT protein variants have been discovered. Nine of them have been reported in the literature and another one (K201 N) is described in the NCBI dbSNP database, with a frequency of 15.7% or heterozygosity of 0.265 out of 51 individuals genotyped (Fig. 1B). I425V is the only one that has been characterized pharmacologically. I425 is located in the middle of hSERT TM8. It is conserved in all eight SERTs listed in Fig. 2 except ceSERT with Phe instead. At the same position, Ile and Val are present in DATs and NETs as well, suggesting—and experimentally confirmed—that mutation I425V would not influence plasma membrane expression of the transporter protein. In vitro assays demonstrated that I425V increases both substrate uptake affinity (K_m values ↓) by 1.2–1.6-fold and V_{max} by 1.7- to 2.2-fold, attributable to the mutation-mediated defect in regulation by nitric oxide and resulting constitutive activation of hSERT (Kilic et al. 2003). This gain-of-function mutant is found in two families with clustering of OCD and other serotonin-related disorders and represents a risk factor for these human diseases (Ozaki et al. 2003).

Of several variants characterized functionally, T4 is conserved in all four mammalian proteins but not in non-mammalian proteins. Since Ser8 with Lys10—conserved in the mammalian proteins as well—is a potential phosphorylation site for protein kinase C, T4 becomes a part of the consensus site "XTXXXS*p*" (S*p* denotes a phosphor-serine) for kinase GSK3 (Fig. 2; Kennelly and Krebs 1991). Therefore, T4 might be important for hSERT regulation and the mutation T4A could result in a dysfunctional transporter protein. L255 in TM4 and L362 in TM7 are both conserved, not only in the SERT but also in the DAT and/or the NET, indicative of their importance in transport activity. Based on this, L255M and L362M conceivably would have altered transporter activity. Another variant, P339L in TM6, might have altered substrate affinity because P339 is highly conserved in all eight SERTs. The other SERT variants, with G56A (N-terminal), K201N (ECL2), E215K (ECL2), S293F (TM5), K605N (C-terminal), and P621S (C-terminal), could have normal transporter activity, either because of their benign locations in the protein or low conservation (Fig. 2).

Ten hSERT coding variants and the hSERT were recently transfected and investigated in parallel to define their total and surface protein expression, antagonist recognition, and transporter modulation by posttranslational, regulatory pathways. Two variants, Pro339Leu and Ile425Val, demonstrated significant changes in surface expression, supporting alterations in 5-HT transport capacity. Each of the SERT variants was capable of rapid, phorbol ester-triggered downregulation, but five variants (Thr4Ala, Gly56Ala, Glu215Lys, Lys605Asn, and Pro612Ser) demonstrated no capacity for 5-HT uptake stimulation after acute protein kinase G (PKG)/p38 mitogen-activated protein kinase (MAPK) activation. Epstein-Barr virus (EBV)-transformed lymphocytes natively expressing the most common of these variants (Gly56Ala) exhibited a similar loss of 5-HT uptake stimulation by PKG/p38 MAPK activators. HeLa cells transfected with the Gly56Ala variant demonstrated elevated basal phosphorylation and, unlike hSERT, could not be further phosphorylated after 8-bromo cyclic guanosine monophosphate (cGMP) (8BrcGMP) treatments. Taken together, spontaneously occurring human SERT coding variants displayed different regulatory patterns when transfected into cell lines. These findings suggest that spontaneous variants of the transporter structure can lead to a diversity of regulatory responses that may confer risks for disorders attributable to compromised 5-HT signaling (Prasad et al. 2005). Whether serotonin affects SERT localization and mobility similarly in various forms of SERT-containing coding region SNPs is a current and notable void in the literature.

2.4 Variations in SERT Expression Levels

hSERT expression levels can be detected by positron emission tomography (PET) imaging of SERT availability or in postmortem tissues by mRNA or protein levels. In different individuals, SERT availability (a measure of density) varied by fourfold as detected by PET imaging, or sixfold as measured by mRNA in postmortem tissue of selected brain regions (Frankle et al. 2005; Little et al. 1998). A number of lines of evidence have linked SERT polymorphisms to SERT expression levels. Several studies have shown that the *l* allele of 5-HTTLPR is associated with higher SERT expression levels. In vitro, a 1.4-kb fragment containing the *l* allele is associated with higher expression levels than one containing the *s* allele in transiently expressed cells (Heils et al. 1996) and in cultured human lymphoblast cell lines carrying the l/l alleles (Lesch et al. 1996). In vivo, the *l*/*l* carriers consistently display twofold higher uptake activity by the SERT in small muscle cells (Eddahibi et al. 2001) and higher mRNA levels in postmortem brain tissues (Little et al. 1998) than the s/s carriers. Imaging studies demonstrate that hSERT availability declines during aging, but there is no evidence for individual differences in the rate of declination (van Dyck et al. 2000).

Altered SERT expression is associated with human diseases, particularly depression and suicide. In healthy individuals (n = 96), SPECT imaging data revealed no correlation between 5-HTTLPR and SERT availability in the brainstem-diencephalon (van Dyck et al. 2004). In a preliminary PET imaging study, hSERT availability was decreased in the midbrain and correlated with severity of major depression (Newberg et al. 2005), possibly indicative of reduced serotonin availability in this brain region. Consistent with these findings was a postmortem study that investigated the association of SERT mRNA with suicide. The suicide group had 54% fewer dorsal raphe nucleus (DRN) neurons expressing SERT mRNA compared with controls, but in the serotonin neurons that expressed the SERT gene, the mRNA level per neuron was greater (Arango et al. 2001), again implying that overactive SERT could reduce serotonin availability. In a different postmortem study of schizophrenic brain, SERT mRNA levels in left superior frontal gyrus were higher but were lower in the left middle temporal gyrus (Hernandez and Sokolov 1997), indicative of a regional specificity of SERT expression levels. In platelets derived from alcoholics, [^{3}H]5-HT uptake and [^{3}H]paroxetine binding assays showed the *l*/*l* carriers displayed higher binding B_{max} values than s/s carriers, consistent with previous findings that *l*/*l* is associated with higher SERT expression. To add to the complexity of these findings, the higher SERT did not correlate with higher transport capacity in the same alcoholic individuals, suggesting that alcoholism may downregulate hSERT function (Javors et al. 2005). Accordingly, transporter density—as well as transporter function—needs to be considered in relating polymorphic regions of the SERT gene to function.

In addition to the 5-HTTLPR, the intron 2 VNTR influences reporter gene expression. Based on a luciferase (Luc) reporter system in vitro that reflects changes in gene expression, the 12-repeat allele of the intron 2 VNTR supported several-fold higher Luc expression than the 10-repeat allele in embryonic stem cells (Fiskerstrand et al. 1999). In order to compare the relative significance of the STin2.10 versus STin2.12 alleles for promoter regulation in vivo, two reporter plasmids were constructed by tagging the two alleles to the 5′-end of an hβg-LacZ hybrid (human β-globin minimal promoter sequence-βgal gene). These two constructs were then introduced into mice by transgenic technology and the βgal activity was examined in the transgenic mice at different developmental stages. At embryonic day (E)10.5, the STin2.12 mice expressed much higher βgal activity in the midbrain and rostral hindbrain floor plate than the STin2.10 mice (MacKenzie and Quinn 1999), indicating that the STin2.12 was a more influential modifier of promoter regulation. Further analysis demonstrated that the transcription factor YB-1 binds to this VNTR to enhance the 12-repeat-associated higher promoter activity in vitro (Klenova et al. 2004). Accordingly, differential allelic interactions with transcription factors may underlie allele-specific promoter activity.

2.5 SERT Association with Human Diseases

The SERT gene has been investigated in an astonishing 330-plus association studies with more than 30 different human diseases. Collectively, more than 10 diseases are positively associated with several markers on the SERT gene, including the promoter 5-HTTLPR and intron 2 VNTR (STin2), as seen in Table 4.

The association of 5-HTTLPR with psychiatric disorders (Table 4) is consistent with the principal involvement of hSERT in regulating serotonergic neuronal activity. The robustness of these association studies benefited from that fact that 5-HTTLPR is a functional polymorphism that significantly influences promoter activity (Heils et al. 1996). Meta-analysis of data from 26 different studies suggests that the SERT gene is a risk factor for anxiety-related traits (Lesch et al. 1996; Schinka et al. 2004). Individuals carrying s/s are more prone to acquire a conditioned fear response (Garpenstrand et al. 2001), which is reflected by the blood oxygen level-dependent (BOLD) functional magnetic resonance imaging (fMRI)-based clinical observations, suggesting that the right amygdala of *s*/*s* carriers displays greater response to fearful stimuli (Hariri et al. 2002). The association of SERT polymorphisms to major depression was anticipated because serotonin-selective reuptake inhibitors (SSRIs) are widely used in treating depression. Indeed, the association between depression and the 5-HTTLPR or the functional intron 2 VNTR has been confirmed in the majority of studies (Furlong et al. 1998; Lotrich and Pollock 2004; Ogilvie et al. 1996). Imaging-based morphometric and functional analyses have demonstrated that *s* allele carriers have reduced gray matter volume in the perigenual cingulate and amygdala, decreased coupling of the amygdala-cingulate feedback circuit (implicated in extinction of negative effect) and impaired emotion regulation (Pezawas et al. 2005). These findings provide biological evidence for SERT variants as a risk factor for major depression.

The *l* allele appears associated with a number of diseases as well. In a recent clinical study in France and Great Britain, *l*/*l* carriers, associated with higher levels of SERT expression in pulmonary artery smooth muscle cells than *l*/*s* and *s*/*s* carriers, displayed higher pulmonary artery pressure and were at higher risk for pulmonary hypertension in chronic obstructive pulmonary disease (Eddahibi et al. 2003). Another consistent finding is association of the *l* allele with ADHD (Bobb et al. 2005a, b; Curran et al. 2005).

5-HTTLPR, VNTR, and other SERT gene markers may or may not collectively show positive association with diseases. Both 5-HTTLPR and 5′ haplotypes are positively associated with autism and smoking and both 5-HTTLPR and VNTR are associated with aggression. In contrast, the 5-HTTLPR, but not VNTR, is positively associated with major depression (Lasky-Su et al. 2005). For schizophrenia, only VNTR, but not 5-HTTLPR, has shown a positive association, whereas for ADHD only the haplotypes, but not 5-HTTLPR, are positively

associated. The missense mutant I425V and the exon 15 SNP 2892G/T are positively associated with OCD. Conceivably, there is low LD between 5-HTTLPR and other markers of the SERT in certain populations. A mechanism-based model of the relationship between SERT function and these neuropsychiatric disorders awaits insight into the functional consequences of each of these variants.

It is important to point out that association studies in different populations can be frequently inconsistent. For example, the "*s*" allele is associated with smoking (Gerra et al. 2005) and consistent with decreased SERT expression in platelets of African-American smokers (Patkar et al. 2003). However, haplotype analysis of 5-HTTLPR and VNTR indicates that that "*l*"+STin2.12, or each of these alleles, is associated with higher SERT expression in vitro and is associated with smoking in a Jewish cohort (Kremer et al. 2005). In another example, 5-HTTLPR, but not the VNTR, is associated very significantly ($p = 3.07\times10^{-5}$) with diarrhea-predominant irritable bowel syndrome [dPIBS] in Caucasian women from North America (Yeo et al. 2004), with the *s* allele over-represented in the patients with an odds ratio (OR) of 2.23. This is consistent with the findings that decreased SERT expression is associated with dPIBS in Italians (Bellini et al. 2003), but not in a Korean (Lee et al. 2004) or a Turkish population (Pata et al. 2002). A U.S. group reported the "*s*" allele association with autism (Cook et al. 1997) but the opposite association with autism ("*l*" allele) was reported by a German group (Klauck et al. 1997). Even for major depression, SERT may not be an essential risk factor (Anguelova et al. 2003). For OCD, the "*l*" allele of 5-HTTLPR was positively associated in two U.S. studies (McDougle et al. 1998) but not in South African, Israeli, German, and Canadian studies (Billett et al. 1997; Frisch et al. 2000; Kinnear et al. 2000; Walitza et al. 2004). In a final example, "*s*" association with anorexia nervosa was observed in both Japanese and French, but not in British Caucasian females (Fumeron et al. 2001; Matsushita et al. 2004; Sundaramurthy et al. 2000). These findings point to the complexity of polygenic diseases that are modulated by ethnic genotype, phenotype, and environment.

3
The Human Norepinephrine Transporter (*SLC6A2*)

3.1
Introduction

Central and peripheral norepinephrine is implicated in a range of physiological functions. Arousal, attention, memory, and mood, as well as autonomic regulation of heart rate and blood pressure, fall within the purview of norepinephrine function. Released norepinephrine in central and peripheral synapses is terminated by the NET, which regulates noradrenergic receptor-mediated neurotransmission. Dysfunction of the NET in cardiac sympathetic

nerve terminals is implicated in hypertension, diabetes, cardiomyopathy, and congestive heart failure (Bohm et al. 1995; Esler et al. 1981; Garland et al. 2002; Mao et al. 2004; Merlet et al. 1992; Shannon et al. 2000). Compromised NET function has been suspected in mood disorders and in ADHD, although the latter association is controversial (Bobb et al. 2005a; Klimek et al. 1997; Xu et al. 2005; Yang et al. 2004). Centrally, the NET is a target of anti-hyperactivity and antidepressant medications as well as psychostimulant drugs of abuse (cocaine, amphetamines).

3.2 NET Genomic VARIANTS

The NET gene is smaller than the DAT gene (47.4 kb). Once again, the NET genomic DNA sequence displays a high density of polymorphisms (~4/KB). More than 200 SNPs are located in this gene (from exons 1 to 15) and more than 40 SNPs are located in the 5′ 10-kb region (the upstream gene *FLJ20481* is 74 kb away from exon 1). Approximately 33 polymorphisms have been documented in the literature and most of them have been included in the dbSNP.

3.2.1 Non-coding Region Variants

One of the non-coding region variants, designated NETpPR, covers 363 bp between −3935 bp and −4297 upstream of exon 1 (Urwin et al. 2002). NETpPR contains six "AAGG"-repeat islands (AAGG1–6) and each island has 3–4 repeats. AAGG1 has 3/4 repeats (*s*/*l* alleles) and AAGG4 has 2/3 repeats (*s*/*l* alleles and *s* has a minor allele frequency (MAF) of 26% in Japanese). Both of the *s* alleles lose the consensus site for transcription factor Elk-1, but these two alleles have not yet been found on the same chromosomes. In Japanese populations, the AAGG4 *l* allele has been found to double the risk for anorexia nervosa (restrictive subtype) as seen in Table 5, but its frequency in other populations has not yet been reported. In some individuals, the 343 bp at the 3′ side of the 363 bp NETpPR can be deleted (Kim et al. 1999; Urwin et al. 2002). Accordingly, the NET gene has a highly polymorphic promoter region and is subject to extensive variations among individuals. Conceivably, some of these variations confer risk factors for NET-related diseases.

Certain polymorphisms either have been used in association studies or are known to cause missense mutations (indicated in the parentheses) in the hNET protein (Fig. 1C). rs3760019, rs2397771, and rs168924, located between NETpPR and exon 1, are T/C, C/G, and A/G SNPs and have been used in a single association study (Ono et al. 2003). Originally designated as T-182C, rs2242446 is located at 131 bp to the 5′-end of exon 2. With an MAF of 25.4% for the C allele, this stretch has been assessed in three association studies (Table 5; Inoue et al. 2004; Ryu et al. 2004; Zill et al. 2002). The intron 9C/A SNP was originally

Table 5 *SLC6A2* association with human diseases

Marker	Diseases	Association (No. of studies)		Reference(s)
5′ region				
NETpPR	Anorexia nervosa (restrictive subtype)	Yes	(1)	Urwin et al. 2002
rs168924	Hypertension	Yes	(1)	Ono et al. 2003
rs2242446	Major depression	Yes	(2)	Inoue et al. 2004; Ryu et al. 2004
		No	(1)	Zill et al. 2002
3′ region				
1429G/C (A457P)	Orthostatic intolerance	Yes	(1)	Shannon et al. 2000
Intron 8 SNP rs3785157	ADHD	Yes	(1)	Bobb et al. 2005
		No	(1)	McEvoy et al. 2002
Intron 10 SNP rs998424	ADHD	Yes	(1)	Bobb et al. 2005
		No	(2)	De Luca et al. 2004; McEvoy et al. 2002

an intron 8 SNP, without considering the discovery of the new exon 1 (Kim et al. 1999; Ono et al. 2003). Intron 9C/A was not associated with hypertension in Japanese (Ono et al. 2003) and has not yet been used for other populations. rs5569, located in exon 10 and originally reported as 1287G/A, has an MAF of 30%–35% for the A allele (Stober et al. 1996; and dbSNP Build 124) and has been used in four association studies (see Sect. 3.5).

LD between SNPs located in intron 1/intron 2 and the SNP rs5569 in exon 10 displays great diversity and varies significantly from subregion to subregion within the gene and from population to population as well. The D′ value is 0.259 in 60 Caucasians for LD between rs2242446 located 0.1 kb before exon 2 and rs5569 in exon 10. rs2242446 has not been genotyped, and no D′ information is available yet for other populations (HapMap). The D′ value for LD between an intron 2 SNP rs3785143 located 4 kb after exon 2 and the exon 10 SNP rs5569 is 0.2 for 60 Caucasians, but it is 1.0 for 45 Chinese, 44 Japanese, and 59 Africans. The D′ value for another region—between the same intron 2 SNP and an intron 11 SNP (rs36009, 0.1 kb 5′ to exon 12)—is 1.0 for Caucasians and Africans, 0.06 for Chinese, and 0.355 for Japanese. A recent systematic haplotype analysis using 26 SNPs across this gene for 384 individuals of Finnish-Caucasian, American-Caucasian, Plains American Indian, and African-American populations showed that there are three high-LD blocks of haplotypes that are conserved among these populations (Fig. 1C; Belfer et al. 2004). The D′ value for a 19.5-kb 5′ region—between the 5′ SNP

rs4783899 (located at 3 kb upstream of exon 1) and the intron 4 SNP rs187714 (0.5 kb after exon 4)—is 0.95, 0.93, 0.99, and 0.81 for the four populations, respectively. However, the frequency of the most common haplotype in each block varies by up to 4- to 5-fold from population to population. The D′ value for LD between the same 5′ SNP and any of other 18 SNPs located downstream of rs187714 is less than 0.12, less than 0.45, less than 0.39, and less than 0.24, respectively. It seems that there are recombination hot spots within intron 4.

3.2.2 Coding Region Variants

Similarly, the coding region of *SLC6A2* displays great diversity as well, and the DNA sequences are more variable than *SLC6A3* and *SLC6A4*. More than 200 individuals have been re-sequenced and 20 missense mutants have been described; the frequency of the variants can be as high as 5.5% (Table 1). This information might suggest that human survivors are more tolerant to hNET-related diseases than to hDAT or hSERT-related diseases.

3.3 NET Protein Variants

hNET has the largest variability in amino acid sequence among the three transporters in question here. Of the 20 protein variants that have been discovered, 15 of them are reported in the literature and the other five (N7K, A31P, V247I, T283R, and I549T) described in dbSNP (Fig. 1C). Sixteen of them have been characterized pharmacologically.

The orthostatic intolerance-associated A457P mutation in TM9 disrupts plasma membrane expression not completely but dominantly to the wildtype and almost eliminates uptake activity based on in vitro analyses (Hahn et al. 2003; Shannon et al. 2000). A457 is highly conserved among the five NETs listed in Fig. 2 and is probably important for norepinephrine transport. The other residues at this position throughout DATs and SERTs are either Ser or Gly with small side-chains. Substitution with the bulky side-chained Pro for this Ala likely changes not only the substrate affinity but, more importantly, the protein conformation, leading to loss of uptake activity.

A similar mutant A369P located in ECL4 loses both plasma membrane expression and uptake activity completely (Hahn et al. 2005). A369 is conserved in neurotransmitter transporters except a Ser residue in ceDAT and msSERT. These findings are consistent with the notion that mutations of conserved residues in this ECL4 often disrupt transporter expression (Lin and Uhl 2003; Lin et al. 1999). R121Q, N292T, and Y548H all lose 22%–44% uptake activity with decreases accordingly in plasma membrane expression. R121 in intracellular loop 1 (ICL1) and Y548 in ECL6 are both highly conserved and might contribute to transporter configuration and/or expression. N292 is present only in the mammalian NETs and is probably important for NET-related transporter

features.

Two of the characterized variants appear to be gain-of-function mutants. F528C displays a 31% increase in [^{3}H]norepinephrine uptake rate compared to the wildtype, apparently through an increase in plasma membrane expression. F528 is conserved throughout the transporters with the exception of an Ile residue in ceDAT. Removal of the phenyl ring from this Phe caused an almost tenfold decrease in uptake V_{max} without influencing expression in DAT (Lin et al. 1999), suggesting the importance of F528 for transport activity. G478S increased uptake affinity (K_m values ↓) by 2.5-fold and displayed an average V_{max}/K_m value of 65, comparing to 175 of the wildtype. G478S represents another gain-of-function mutant (Runkel et al. 2000). G478 in TM10 is conserved in 89% of the transporters or all of the mammalian transporters and, at the same position, an alternative is Ser in ceSERT or Thr in ceDAT (Fig. 2). G478 is apparently important for norepinephrine translocation.

Variants V69I, T99I, V2441I, V245I, V356L, N375S, V449I, and I549T appear to have normal transport activity (Hahn et al. 2005; Runkel et al. 2000). None of them influences uptake activity at statistically significant levels. V245I increased desipramine affinity by 2.8-fold. V449I increased the V_{max}/K_m value by 1.6-fold and desipramine affinity by 2.4-fold (Runkel et al. 2000). V69 is located in TM1 and highly conserved among the transporters (Fig. 2). It is little surprising that Ile substitution did not influence the uptake activity and increased desipramine affinity by 1.4-fold.

Among the four uncharacterized hNET variants (Fig. 1C), N7 and A31 are located in the N-terminus and not conserved among NETs. It would be surprising to see any functional alteration in N7K and A31P. At position V247 in TM4, Ile is an alternative, and V247I probably has normal transport activity as well. T283 in TM5 is conserved in all of the mammalian transporters. T283R has a substitution with a positively charged large side-chain and is thus expected to have altered transport activity. All of the speculations concerning these four variants, especially T283R, need to be verified by experimental studies.

3.4 Variations in hNET Expression Levels

There is some information available about genotypic association with hNET expression levels. Variations in hNET expression may be a significant factor in consideration of ADHD treatment strategies (Fischman and Madras 2005; Madras et al. 2005). It has been reported that expression levels in placenta vary widely from individual to individual and lower mRNA levels are associated with pre-eclampsia (Bottalico et al. 2004; Szot et al. 2000). Brain imaging data are not available currently due to lack of appropriate labeling agents (McConathy et al. 2004). Based on this summary, the human genetics of *SLC6A2* warrants further investigation.

3.5
NET Association with Human Diseases

There are approximately 20 association studies reported for the NET (Table 5). Findings from these studies suggest that this gene is associated with major depression, hypertension, orthostatic intolerance, and anorexia nervosa (restrictive type). The intron 8 and 10 SNPs rs3785157 and rs998424 were positively associated with ADHD in a U.S. study, but not in Canadian and Irish studies (Bobb et al. 2005a, b; De Luca et al. 2004; McEvoy et al. 2002).

The exon 10 SNP rs5569 (1287G/A) was not associated with major depression in Caucasian Germans and Canadians (Owen et al. 1999; Zill et al. 2002), personality disorders in Chinese (Tsai et al. 2002), nor bipolar disorder or schizophrenia in Poles (Leszczynska-Rodziewicz et al. 2002). This SNP—82 bp away from and probably in strong LD with the A457P mutant—has not yet been examined for potential association with orthostatic intolerance. None of the five SNPs rs1805064 (V69I), rs1805065 (T99I), rs1805066 (V245I), rs2234910 (V449I), and rs1805067 (G478S) was associated with panic disorder, Tourette's syndrome, bipolar disorder, or schizophrenia in Caucasian Germans (Sand et al. 2002; Stober et al. 1999; Stober et al. 1996). Because of the low LD between the 5′- and the 3′-ends, markers located in each of the three blocks of high LD should be used in future association studies (Belfer et al. 2004). Markers including NETpPR in the 5′ promoter region merit investigation for ADHD because hNET is a therapeutic target for this disease (Madras et al. 2005).

4
Transporter Gene Knockout Mice: Implications

Studies in mice with full or partial deletions of transporter genes have provided important insights into the genes' contributions to neurochemistry, morphology, and behavior. Null mutations of the DAT gene are not lethal, suggesting that humans can sustain significant variability in hDAT expression levels. However, null mutant mice (DAT −/−) display profound adaptive phenotypic changes, including reduced D1 and D2 DA receptor expression levels in basal ganglia, decreased tissue DA concentrations, increased extracellular DA concentrations, and retarded pituitary development. Mice with null mutations (−/−)—but not heterozygous cohorts (+/−)—display higher locomotor activity, anterior pituitary hypoplasia and dwarfism, and unresponsiveness to the locomotor stimulant effects of cocaine, but they retain cocaine self-administration unless the serotonin transporter is also deleted (Bosse et al. 1997; Giros et al. 1996; Sora et al. 2001; Sora et al. 1998). Mice lacking SERT display normal development, reduced 5-HT concentration, and increased locomotion activity in response to (+)-3,4-methylenedioxymethamphetamine ("ecstasy") treatments (Bengel et al. 1998). The DAT and SERT double knockouts, but none of the single knock-

outs, lose cocaine-induced place preference, suggesting that DAT and SERT are important for cocaine reward (Sora et al. 2001). Mice lacking NET have increased extracellular norepinephrine concentration, reduced norepinephrine tissue concentrations, and reduced extracellular DA concentrations. The −/− mice display supersensitivity to sensitization by amphetamine and cocaine treatments (Xu et al. 2000). Interestingly, none of the heterozygote mice displays any significant behavioral alterations.

5 Summary

Neurotransmitter transporters are principal regulators of neurotransmission. Genomic variations in these transporter genes may cause missense mutations in the proteins and alterations in expression levels in a brain region-specific manner, which confer risk factors for many related human diseases. The involvement of multiple endogenous and exogenous factors in pathogenesis of human diseases adds a level of complexity to fundamental transporter genomics. Genetic manipulation of these transporter genes in rodents can facilitate our understanding of the biological and pharmacological consequences of changes in transporter gene function, but cannot fully replicate human environmental and other factors. Considerable progress has been made in identifying genetic variations and relating these variations to biological function, pharmacological response, and human diseases during the last decade. Our current knowledge will guide future strategies to identify population-specific and environmental risk factors. Because of the low LD between discrete regions in the genes, particularly *SLC6A3* and *SLC6A2,* and variable frequency of these variations among different populations, numerous challenges remain. In addition to the few described herein, of which hundreds of polymorphisms in each gene are functionally important, questions remain concerning which haplotypes carry functionally relevant alleles and confer risk factors for diseases. Future research will provide the infrastructure to develop a comprehensive model of the contribution of haplotypes, other involved genes, population differences, and cultural and environmental influences in engendering pathophysiological states of polygenic disease and drug response.

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