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ORIGINAL RESEARCH ARTICLE

Polymorphisms in the 3′-untranslated region of human and monkey dopamine transporter genes affect reporter gene expression

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Dopamine transporter (DAT) levels vary in normal subjects and deviate from the normal range in pathological states. We investigated mechanisms by which the DAT gene may influence DAT protein expression. As the 3′-untranslated region (3′-UTR) of the DAT gene varies with regard to length and single nucleotide polymorphisms (SNPs), we addressed whether the 3′-UTR of sequence-defined DAT alleles can differentially affect the level of reporter gene expression *in vitro*. We first established that within individual rhesus monkeys, two alleles of the DAT gene were expressed in the substantia nigra. We then transfected HEK-293 cells with HSV-TK- and SV40-driven luciferase expression vectors harboring downstream DAT 3′-UTR segments of alleles containing polymorphisms of length (human: 9 or 10 repeat units) or SNPs within alleles of fixed length (human: *DraI*-sensitive (DraI+) *vs DraI*-insensitive (DraI–) 10-repeat alleles; rhesus monkey: *Bst1107I*-sensitive (Bst+) *vs Bst1107I*-insensitive (Bst–) 12-repeat alleles). Vectors containing the 3′-UTR segment of a human DAT allele containing nine tandem repeat units resulted in significantly higher levels of luciferase production than analogous vectors containing 10 tandem repeat units. Depending on the promoter used, vectors containing the human or monkey 3′-UTR segments that differed on the basis of an SNP resulted in increases or decreases in luciferase gene expression. This report provides experimental evidence that variability in the length or the sequence of the 3′-UTR of the DAT gene may influence levels of DAT protein in the brain.
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Introduction

The brain dopamine transporter (DAT) is a member of a superfamily of Na^+/Cl^- dependent neurotransmitter transporters.[1–3] By actively sequestering extracellular dopamine to intracellular compartments, the DAT plays a key role in adjusting dopamine availability and consequent dopamine-mediated behaviors. As dopamine is implicated in substance abuse and neuropsychiatric or neurodegenerative diseases, transporter levels and function may contribute to the etiology of or susceptibility to dopamine-related disorders. DAT protein levels vary in normal subjects, particularly as a function of age, and deviate from the normal range in pathological states.[4–8] DAT protein is markedly reduced in Parkinson's disease,[9,10] Lesch–Nyhan syndrome[11] and reportedly is elevated in attention deficit hyperactivity disorder (ADHD),[12–14] Tourette's syndrome,[15] and major depression.[16] Chronic use of stimulant drugs such as cocaine leads to increases in DAT levels during withdrawal whereas methamphetamine results in DAT depletion, possibly a consequence of neurotoxicity.[17–21]

Individual variability and deviation from normal ranges of DAT levels may be attributable to a variety of factors ranging from degeneration of dopamine neurons, hyperinnervation, to drug-induced neuroadaptive processes. Diversity within the DAT gene (Figure 1) may also influence DAT protein levels expressed in brain. The coding region of the DAT gene is relatively invariant and infrequently contains polymorphisms or mutations that are associated with dopamine-related disorders.[22–24] The discovery of a variable number of tandem repeat sequences (VNTR) in the 3′-UTR of the DAT gene[25] broadened the scope of potential sources of DAT diversity.[26–28] Research to link the number of tandem repeat units in the VNTR (ie, the length of the 3′-UTR) with dopamine-related disorders uncovered an association between alleles with the 10-repeat length and ADHD[29–33] (but see Holmes *et al*[34]). Less consistently, alleles with the 9-repeat length have been associated with cocaine-induced paranoia and alcohol or nicotine addictions.[35–42]

Emergent from these findings is whether the number

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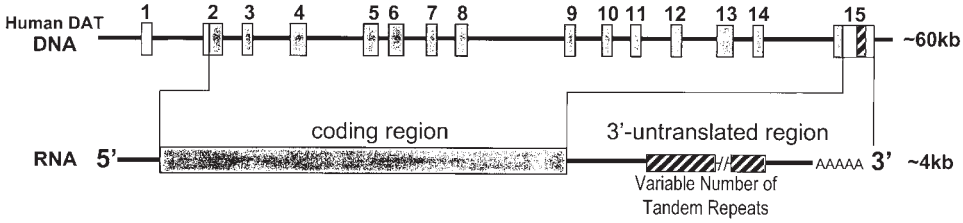

Figure 1 Schematic representation of the human dopamine transporter gene. Shaded exons in the DNA form the coding region, white exons contribute to the 5′ and 3′ non-coding regions. The 3′-untranslated region of the RNA is derived from a major portion of exon 15 and includes a variable number tandem repeat (VNTR) region (stripes). The number of 40-bp repeat sequences varies between three and 13 copies, resulting in alleles of the dopamine transporter with different lengths.

of repeat sequences in the 3′-UTR of the DAT gene influences DAT protein levels in the brain. To investigate the relationship between genotype and phenotype, two studies measured DAT levels in living human brain striatum with single photon emission computed topography (SPECT) and genotyped DAT alleles in the same subjects by the number of tandem repeats in the VNTR region. Opposite findings were reported as subjects with the 9-repeat/10-repeat genotype either had lower[38] or higher[43] DAT levels compared with subjects containing two 10-repeat alleles. This apparent discrepancy may arise for a number of reasons, including the existence of allele diversity independent of the length of the DAT 3′-UTR. In investigating this region of the DAT gene, we and others previously identified novel SNPs in the 3′-UTR of human and rhesus monkey DAT genes.[22–24,44,45] Based on these findings, we proposed that SNPs in the 3′-UTR of the DAT gene may be another factor that underlies variability in DAT protein levels.[44]

We now report the impact of polymorphisms either of variable length or single bases in the DAT 3′-UTR on levels of reporter gene expression *in vitro*. The results demonstrate that length and nucleotide variations in DAT 3′-UTR segments modify reporter gene expression and may contribute to the diversity of DAT expression between individuals. The biological relevance of these variations is sustained by the present finding that two alleles of the DAT gene are expressed as mRNA in the substantia nigra of brain in individual monkeys. Further investigation of the relevance of the DAT gene 3′-UTR to the etiology of dopamine-related disorders is warranted. A preliminary report of the research was presented at the 30th Annual Meeting of the Society for Neuroscience (New Orleans, LA, USA, 2000).

Materials and methods

Cloning of DAT 3′-UTR segments

The methods for cloning segments of the human 10-repeat DAT 3′-UTRs and the rhesus monkey 12-repeat DAT 3′-UTRs have been previously described.[44] A nine-repeat DAT 3′-UTR segment derived from human genomic DNA was cloned and the sequence is now reported. Briefly, polymerase chain reaction (PCR) was carried out in 30 μl total volume containing 50 ng human genomic DNA (Promega, Madison, WI, USA, catalog No. G1471 and G1521), 10 pmol of each primer (U10 and L13),[44] 2.5 U Red Taq (Sigma, St Louis, MO, USA), 3 μl 10× Red Taq buffer (Sigma), 333 μM dNTP (Life Technologies, Rockville, MD, USA) and water to volume. Thermal cycling included: (1) 94°C for 1 min; (2) 94°C for 12 s; (3) 53.5°C for 30 s; (4) 72°C for 165 s; (5) 39 cycles, steps 2, 3 and 4; and (6) 72°C final extension for 15 min. The reaction was performed in an MJ DNA engine PTC 200 thermocycler (MJ Research, Waltham, MA, USA). The PCR product was isolated from a 1% agarose gel and purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA). Isolated DNA was cloned into pcDNA3.1/V5/His-TOPO (InVitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Following transformation, bacterial colonies were reserved and screened for insert orientation by direct PCR using a T7 forward primer and the reverse amplimer used for cloning. A colony with positive PCR results was selected for plasmid DNA isolation with a Wizard miniprep kit (Promega) according to the manufacturer's protocols.

Expression of alleles in monkey substantia nigra

Brain tissue was harvested from three adult female monkeys that were euthanized in the course of other studies. The substantia nigra was dissected and frozen for less than 1 year at –80°C. For RNA and DNA extraction, each substantia nigra was removed from –80°C, immediately placed in RNA*later*™ solution (Ambion, Austin, TX, USA) and stored at 4°C for less than 1 week. RNA was extracted from homogenized tissue (Dounce Tissue Grinder, Kontes, VWR Scientific) using an RNA Isolator kit (RNA-ISO-050, Sigma Genosys, The Woodlands, TX, USA) according to the manufacturer's protocol. All RNA samples were then treated with RQ1 RNase-free DNase (Promega) to remove potential genomic DNA contamination present in the RNA samples. Genomic DNA was isolated from the same homogenate and was ultra-purified using the Wizard® DNA Cleanup System (Promega). RNA samples were then subjected to a reverse transcriptase reaction with (+RT) and without (–RT) Superscript II RNase H^- reverse transcriptase (Life Technologies), according to the manufacturer's protocols. PCR reactions were then carried out on each of the –RT, +RT and genomic DNA samples using primers U13 and L12.[44] After cycling, PCR products were purified using a Qiaquick™ PCR clean-up kit (Qiagen). Purified DNA was eluted in 15 μl of RNase-free, DNase-free water

(Sigma), and digested with 0.5 μl of the restriction enzyme, *Bst1107I* (8–12 U μl^{-1}, Roche Molecular Biochemicals, Indianapolis, IN, USA) in a total volume of 20 μl for 1–2 h at 37°C. Samples were loaded on a 2.5% agarose gel containing ethidium bromide for electrophoresis at 120 V for 1 h. Gels were visualized and documented under UV transillumination.

Reporter vector construction

Human DAT 3′-UTR segments were initially cloned into pcDNA3.1/V5-His/TOPO (InVitrogen). Each pcDNA3.1/V5-His/TOPO-DAT-3′-UTR vector was linearized with BamHI, blunted with T4 DNA polymerase, and cut with HindIII. A firefly luciferase cDNA (Luc) was excised from pSP-Luc(+) (Promega) with HindIII and EcoRV and was subcloned upstream from the DAT 3′-UTR segments. The resulting vectors were digested with HindIII and PmeI, liberating Luc-DAT 3′-UTR cassettes. pRL-SV40 and pRL-TK (Promega) were digested with BamHI, blunted with T4 DNA polymerase and digested with HindIII. The resulting vector backbones had only an ampicillin resistance gene, an origin of replication and either the SV40 or HSV-TK promoter. Each Luc-DAT3′-UTR cassette was then directionally cloned into both pRL-SV40 and pRL-TK vector backbones.

The rhesus monkey DAT 3′-UTR segments were initially cloned into pGEM-T-easy (Promega) and were then excised with NotI. Luc was excised from pSP-Luc(+) (Promega) with NheI and XhoI and was cloned into those sites in pcDNA3.1(–) (InVitrogen). This vector (pcDNA3.1-Luc) was linearized with NotI and each monkey DAT 3′-UTR segment was cloned into the vector. Insert orientation was determined by PCR on bacterial colonies using a T7 forward primer and GL2 primer (Promega). Selected vectors were then digested with BamHI and NheI, liberating Luc-DAT3′-UTR cassettes. pRL-SV40 and pRL-TK (Promega) were digested with BamHI and NheI, and the Luc-DAT3′-UTR cassettes were directionally cloned into the vector backbones. The methods for construction of human and monkey vectors were slightly different, resulting in the retention of a chimeric intron in the monkey but not the human vectors. However, all human and all monkey vectors sharing the same promoter differed only within their respective DAT 3′-UTR sequences.

DNA sequencing

Sequencing of both strands of all 3′-UTRs was performed using a PE Applied Biosystem 373 automated DNA sequencer and Bigdye dye-terminator cycle sequencing methodology according to the protocol of the manufacturer (Perkin-Elmer, Norwalk, CT, USA). The Molecular Biology Core Facilities, Dana Farber Cancer Institute, Boston, Massachusetts performed sequencing.

Transient transfection and luciferase assays

HEK-293 cells (American Type Culture Collection, Manassas, VA, USA) were grown in 145-mm untreated tissue culture dishes (Greiner America, Lake Mary, FL, USA) in growth medium consisting of DMEM supplemented with 10% fetal bovine serum, 100 U ml^{-1} penicillin, 100 μg streptomycin, and 0.1 mM non-essential amino acids (all reagents from Life Technologies), at 5% CO_2 in a 37°C water-jacketed incubator. For luciferase assays, cells were replated in 12-well tissue culture plates (Costar, 07–200–82, Fisher Scientific) in 500 μl $well^{-1}$ growth medium. On the following day, an Effectine Reagent kit (Qiagen) was used for DNA transfection procedures. Cells were transfected at 40–80% confluence with the addition of 475 μl of the following mixture: 9 μl DNA (1 μg μl^{-1}), 9.6 μl Enhancer, 281 μl EC Buffer, 24 μl Effectine reagent and 1.6 ml growth medium in accordance with the manufacturer's protocol. Transfection media was not removed until aspirated at the time of assay. For all experiments, treatments were performed in triplicate wells and in parallel with other vectors of the same class (all vectors sharing the same promoter and 3′-UTRs of the same species). Experiments were repeated three to six times (Table 1), with different DNA preps of the same clones to insure validity.

Transiently transfected cells were assayed for luciferase activity on the day following transfection. Luciferase assays were carried out using the Luciferase Assay System (E1500; Promega). Each well was aspirated and cells were lysed in-well in 500 μl 1× Reporter Lysis Buffer. The plate was placed on an orbital shaker and gently rotated for 10 min. Lysates were collected into 1.5-ml microfuge tubes, vortexed for 10 s and centrifuged for 2 min at 12 000 rpm. Lysate (20 μl) was carefully removed from each microfuge tube and assayed in a Berthold Lumat LB 9507 Luminometer (Perkin Elmer Wallac, Gaithersburg, MD, USA). The injected volume of Luciferase Assay Substrate was 100 μl, the delay setting was 2 s and the measuring time was 10 s. All relative light unit (RLU) counts were in the linear range of $>10^3$ to $<10^6$. Variability in the magnitude of the RLU counts between experiments was evident and possibly influenced by differences in the initial plating density of cells. Therefore, for each experiment, data were normalized as percent of values derived concurrently from the 10-repeat DraI+ values (human) or the 12-repeat Bst+ values (rhesus monkey), which were considered 100%.

Statistics

ANOVA was performed on data derived from each subset of vectors that share a common promoter and contain 3′-UTRs from the same species. Tukey's post hoc comparisons determined significant differences between different vectors.

Genbank accession numbers

AF325530 Homo sapiens dopamine transporter 3′ untranslated region, 9-repeat allele.
AF287465 Homo sapiens dopamine transporter 3′ untranslated region, DraI-sensitive 10-repeat allele.
AF287466 Homo sapiens dopamine transporter 3′ untranslated region, DraI-insensitive 10-repeat allele.

Table 1 Percent normalized relative light units (RLU) for each experiment. These data are summarized in Figure 4

Vector	*Rhesus monkey*				*Human*					
	HSV-Tk-Luc Bst1107I+ (%)	*HSV-TK-Luc Bst1107I– (%)*	*SV40-Luc Bst1107I+ (%)*	*SV40-Luc Bst1107I– (%)*	*HSV-TK-Luc 10-repeat DraI+ (%)*	*HSV-TK-Luc 9-repeat (%)*	*HSV-TK-Luc 10-repeat DraI– (%)*	*SV40-Luc 10-repeat DraI+ (%)*	*SV40-Luc 9-repeat (%)*	*SV40-Luc 10-repeat DraI– (%)*
Experiment 1	100	142.63	100	45.51	100	258.84	135.96	100	77.38	74.38
Experiment 2	100	102.63	100	85.11	100	227.92	165.39	100	143.45	44.36
Experiment 3	100	134.56	100	47.06	100	162.63	125.07	100	108.35	39
Experiment 4	100	140.39	100	30.55	100	132.6	112.07	100	114.31	
Experiment 5			100	40.17	100	188.92	119.89	100	176.6	
Experiment 6					100	113.74	104.43	100	177.72	
Mean	100	130.1	100	49.7	100	180.8	127.1	100	133	52.6
SEM	0	9.3	0	9.32	0	22.75	8.83	0	16.39	11.01

AF287462 Macaca mulatta dopamine transporter 3′ untranslated region, 12-repeat allele A.
AF287463 Macaca mulatta dopamine transporter 3′ untranslated region, 12-repeat allele B.

Results

Cloning of DAT alleles

We previously reported the sequence of two rhesus monkey DAT 3′-UTR segments that contain 12 tandem repeats and are distinguished by an SNP that affects a *Bst1107I* restriction site.[44] We now compare the sequences of 3′-UTR segments derived from monkey and human. In the human, our cloned portions of the 3′-UTR of the DAT gene contain either a *DraI*-sensitive or a *DraI*-insensitive sequence distal to the 10-repeat VNTR region, or a 9-repeat VNTR region (Figure 2).

Expression of both alleles of the monkey DAT gene in brain

Initially, to investigate the biological relevance of the SNP identified in the monkey genome, it was necessary to verify that both alleles of the rhesus monkey DAT gene were expressed as mRNA in brain. We isolated total RNA and genomic DNA from the substantia nigra of three rhesus monkey brains and compared the two preparations. All samples (+RT, –RT and genomic DNA reactions) were used for PCR and subsequently digested with *Bst1107I* to determine whether a *Bst1107I*-sensitive polymorphism was present in none, one or both alleles of the DAT gene (Figure 3).

Genomic DNA amplification followed by restriction analysis revealed that rhesus monkeys No. 1 and No. 2 were heterozygous, as two bands were visible on the gel in the gDNA lanes. The upper band is a *Bst1107I*-insensitive allele and lower band is a *Bst1107I*-sensitive allele. Animal No. 3 was homozygous, as both DAT alleles present were *Bst1107I*-sensitive, and as indicated by a single lower band on the gel. To control for DNA contamination, RNA was reverse transcribed in the presence (+RT) and absence (–RT) of reverse transcriptase. The absence of detectable signal in the –RT lanes indicated genomic DNA was not a contaminant in the DNase-treated RNA samples. For each animal, an identical pattern was observed for the +RT –PCR and gDNA samples, indicating that both alleles of the DAT gene were expressed as mRNA in the substantia nigra.

Comparison of rhesus monkey DAT 3′-UTRs on luciferase reporter gene expression

As the repeat sequence of the 3′-UTR of monkey DAT gene appeared to be of fixed length,[44] our investigation was restricted to SNPs in the 3′-UTR. We previously cloned 3′-UTR segments of two 12-repeat alleles of the rhesus monkey DAT gene and detected an SNP, sensitive to a *Bst1107I* restriction enzyme.[44] Presently, we compared whether the *Bst1107I*-sensitive and *Bst1107I*-insensitive 3′-UTR segments of the rhesus monkey DAT gene differ in their effects on luciferase reporter gene expression. Luciferase levels were compared in HEK-293 cells transiently transfected with pTK-Luc/*Bst1107I*+ or pTK-Luc/*Bst1107I*–. We also determined whether SNP effects were a function of the particular promoter used, and compared two additional constructs containing the SV40 promoter (pSV40-Luc/*Bst1107I*+ and pSV40-Luc/*Bst1107I*–).

The two rhesus monkey DAT 3′UTR segments had opposite effects on luciferase reporter levels that were dependent upon the promoter in the vector (Figure 4, top). Cells transfected with the pTK-Luc/*Bst1107I*– vector had significantly higher levels of luciferase than cells transfected with the pTK-Luc/*Bst1107I*+ vector (Figure 4, *Bst– vs Bst+*, *a*: $P < 0.05$, $n = 4$). Conversely, cells transfected with the pSV40-Luc/*Bst1107I*– vector had significantly lower levels of luciferase than cells transfected with the pSV40-Luc/*Bst1107I*+ vector (Figure 4, *Bst– vs Bst+*, *b*: $P < 0.01$, $n = 5$). Data from individual experiments are summarized in Table 1.

Comparison of human DAT 3′-UTRs on luciferase reporter gene expression

A similar analysis was performed with human 3′-UTRs of the DAT gene. We investigated whether the length of the repeat sequence and/or SNPs in the 3′-UTR would affect levels of luciferase reporter gene expression. A 10-repeat *DraI*-sensitive DAT 3′-UTR segment, a 10-repeat *DraI*-insensitive DAT 3′-UTR segment and a nine repeat DAT 3′-UTR segment were cloned from pooled human genomic DNA samples. A firefly luciferase cDNA was placed immediately upstream from each 3′-UTR segment and the resulting chimeric reporter genes were excised from the parent vector and subcloned into modified pRL-SV40 and pRL-TK expression vectors containing either the SV40 or HSV-TK promoter, an ampicillin resistance gene and a bacterial origin of replication. The resulting six vectors (pTK-Luc/10-repeat-*DraI*+, pTK-Luc/9-repeat, pTK-Luc/10-repeat-*DraI*– and pSV40-Luc/10-repeat-*DraI*+, pSV40-Luc/9-repeat, pSV40-Luc/10-repeat-*DraI*–) were each transiently transfected into HEK-293 cells, which were assayed for luciferase activity 24 h later.

To compare directly the effects of a 9- or a 10-repeat sequence on protein expression, cells were transfected with the pTK-Luc/9-repeat vector or the pTK-Luc/10-repeat vectors. For the HSV-TK promoter, (Figure 4, bottom left), the pTK-Luc/9-repeat vector had significantly higher levels of luciferase than cells transfected with pTK-Luc/10-repeat-*DraI*+ (Figure 4, *c*: $P < 0.01$, $n = 6$). The pTK-Luc/9-repeat vector also resulted in higher levels of luciferase activity than the pTK-Luc/10-repeat-*DraI*– vector (Figure 4, *c*: $P < 0.05$, $n = 6$). Although the pTK-Luc/10-repeat-*DraI*– vector resulted in higher levels of luciferase activity compared with the pTK-Luc/10-repeat-*DraI*+, results tended towards but did not achieve statistical significance with our analysis (see Table 1).

In parallel with the cells transfected with the HSV-TK promoter-containing vectors, cells transfected with the pSV40-Luc/9-repeat vector had higher levels of luciferase than cells transfected with the pSV40-

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Consensus     CACAAATTCATGCAAAGTCCTTTCCCGATGCGTGGCTCCCAGCAGAGGCCGTAAATTGAGCGTTCAGTTGACACATTGCA
9-repeat      CACAAATTCATGCAAAGTCCTTTCCCGATGCGTGGCTCCCAGCAGAGGCCGTAAATTGAGCGTTCAGTTGACACATTGCA 80
10 DraI +     CACAAATTCATGCAAAGTCCTTTCCCGATGCGTGGCTCCCAGCAGAGGCCGTAAATTGAGCGTTCAGTTGACACATTGCA 80
10 DraI -     CACAAATTCATGCAAAGTCCTTTCCCGATGCGTGGCTCCCAGCAGAGGCCGTAAATTGAGCGTTCAGTTGACACATTGCA 80

Consensus     CACACAGTCTGTTCAGAGGCATTGGAGGATGGGGGTCCTGGTATGTCTCACCAGGAAATTCTGTTTATGTTCTTGCAGCA
9-repeat      CACACAGTCTGTTCAGAGGCATTGGAGGATGGGGGTCCTGGTATGTCTCACCAGGAAATTCTGTTTATGTTCTTGCAGCA 160
10 DraI +     CACACAGTCTGTTCAGAGGCATTGGAGGATGGGGGTCCTGGTATGTCTCACCAGGAAATTCTGTTTATGTTCTTGCAGCA 160
10 DraI -     CACACAGTCTGTTCAGAGGCATTGGAGGATGGGGGTCCTGGTATGTCTCACCAGGAAATTCTGTTTATGTTCTTGCAGCA 160

Consensus     GAGAGAAATAAAACTCCTTGAAACCAGCTCAGGCTACTGCCACTCAGGCAGCCTGTGGGTCCTTGCGGTGTAGGG.ACGG
9-repeat      GAGAGAAATAAAACTCCTTGAAACCAGCTCAGGCTACTGCCACTCAGGCAGCCTGTGGGTCCTTGCGGTGTAGGGTACGG 240
10 DraI +     GAGAGAAATAAAACTCCTTGAAACCAGCTCAGGCTACTGCCACTCAGGCAGCCTGTGGGTCCTTGCGGTGTAGGGAACGG 240
10 DraI -     GAGAGAAATAAAACTCCTTGAAACCAGCTCAGGCTACTGCCACTCAGGCAGCCTGTGGGTCCTTGCGGTGTAGGGAACGG 240
                    ▼                               ▼

Consensus     CCTGAGAGGAGCGTGTCCTATCCCCGGACGCATGCAGGGCCCCCACAGGAGCGTGTCCTATCCCCGGACGCATGCAGGGC
9-repeat      CCTGAGAGGAGCGTGTCCTATCCCCGGACGCATGCAGGGCCCCCACAGGAGCGTGTCCTATCCCCGGACGCATGCAGGGC 320
10 DraI +     CCTGAGAGGAGCGTGTCCTATCCCCGGACGCATGCAGGGCCCCCACAGGAGCGTGTCCTATCCCCGGACGCATGCAGGGC 320
10 DraI -     CCTGAGAGGAGCGTGTCCTATCCCCGGACGCATGCAGGGCCCCCACAGGAGCGTGTCCTATCCCCGGACGCATGCAGGGC 320
                    ▼                               ▼

Consensus     CCCCACAGGAGCATGTCCTATCCCTGGACGCATGCAGGGCCCCCACAGGAGCGTGTACTACCCCAGAACGCATGCAGGGC
9-repeat      CCCCACAGGAGCATGTCCTATCCCTGGACGCATGCAGGGCCCCCACAGGAGCGTGTACTACCCCAGAACGCATGCAGGGC 400
10 DraI +     CCCCACAGGAGCATGTCCTATCCCTGGACGCATGCAGGGCCCCCACAGGAGCGTGTACTACCCCAGAACGCATGCAGGGC 400
10 DraI -     CCCCACAGGAGCATGTCCTATCCCTGGACGCATGCAGGGCCCCCACAGGAGCGTGTACTACCCCAGAACGCATGCAGGGC 400
                    ▼                               ▼

Consensus     CCCCACAGGAGCGTGTACTACCCCAGGACGCATGCAGGGCCCCCACTGGAGCGTGTACTACCCCAGGA~GCATGCAGGGC
9-repeat      CCCCACAGGAGCGTGTACTACCCCAGGACGCATGCAGGGCCCCCACTGGAGCGTGTACTACCCCAGGATGCATGCAGGGC 480
10 DraI +     CCCCACAGGAGCGTGTACTACCCCAGGACGCATGCAGGGCCCCCACTGGAGCGTGTACTACCCCAGGACGCATGCAGGGC 480
10 DraI -     CCCCACAGGAGCGTGTACTACCCCAGGACGCATGCAGGGCCCCCACTGGAGCGTGTACTACCCCAGGACGCATGCAGGGC 480
                    ▼                               ▼

Consensus     CCCCACAGGAGCGTGTCCTATCCCCGGACC                                   GGACGCATGC
9-repeat      CCCCACAGGAGCGTGTCCTATCCCCGGACC----------------------------------------GGACGCATGC 520
10 DraI +     CCCCACAGGAGCGTGTCCTATCCCCGGACCGGACGCATGCAGGGCCCCCACAGGAGCGTGTACTACCCCAGGACGCATGC 560
10 DraI -     CCCCACAGGAGCGTGTCCTATCCCCGGACCGGACGCATGCAGGGCCCCCACAGGAGCGTGTACTACCCCAGGACGCATGC 560
                    ▼                               ▼

Consensus     AGGGCCCCCACAGGAGCGTGTACTACCCCAGGATGCATGCAGGGCCCCCACAGGAGCGTGTACTACCCCAGGACGCATGC
9-repeat      AGGGCCCCCACAGGAGCGTGTACTACCCCAGGATGCATGCAGGGCCCCCACAGGAGCGTGTACTACCCCAGGACGCATGC 600
10 DraI +     AGGGCCCCCACAGGAGCGTGTACTACCCCAGGATGCATGCAGGGCCCCCACAGGAGCGTGTACTACCCCAGGACGCATGC 640
10 DraI -     AGGGCCCCCACAGGAGCGTGTACTACCCCAGGATGCATGCAGGGCCCCCACAGGAGCGTGTACTACCCCAGGACGCATGC 640

Consensus     AGGGCCCCCATGCAGGCAGCCTGCAGACCACACTCTGCCTGGCCTTGAGCCGTGACCTCCAGGAAGGGACCCCACTGGAA
9-repeat      AGGGCCCCCATGCAGGCAGCCTGCAGACCACACTCTGCCTGGCCTTGAGCCGTGACCTCCAGGAAGGGACCCCACTGGAA 680
10 DraI +     AGGGCCCCCATGCAGGCAGCCTGCAGACCACACTCTGCCTGGCCTTGAGCCGTGACCTCCAGGAAGGGACCCCACTGGAA 720
10 DraI -     AGGGCCCCCATGCAGGCAGCCTGCAGACCACACTCTGCCTGGCCTTGAGCCGTGACCTCCAGGAAGGGACCCCACTGGAA 720
                                                                              *
Consensus     TTTTATTTCTCTCAGGTGCGTGCCACATCAATAACA^CAGTTTTTATGTTTGCGAATGGCTTT~TAAAATCATATTTACC
9-repeat      TTTTATTTCTCTCAGGTGCGTGCCACATCAATAACAGCAGTTTTTATGTTTGCGAATGGCTTTTTAAAATCATATTTACC 760
10 DraI +     TTTTATTTCTCTCAGGTGCGTGCCACATCAATAACAACAGTTTTTATGTTTGCGAATGGCTTTTTAAAATCATATTTACC 800
10 DraI -     TTTTATTTCTCTCAGGTGCGTGCCACATCAATAACAACAGTTTTTATGTTTGCGAATGGCTTTCTAAAATCATATTTACC 800

Consensus     TGTGAATCAAAACAAATTCAAGAATGCAGTATCCGCGAGCCTGCTTGCTGATATTGCAGTTTTTGTTTACAAGAATAATT
9-repeat      TGTGAATCAAAACAAATTCAAGAATGCAGTATCCGCGAGCCTGCTTGCTGATATTGCAGTTTTTGTTTACAAGAATAATT 840
10 DraI +     TGTGAATCAAAACAAATTCAAGAATGCAGTATCCGCGAGCCTGCTTGCTGATATTGCAGTTTTTGTTTACAAGAATAATT 880
10 DraI -     TGTGAATCAAAACAAATTCAAGAATGCAGTATCCGCGAGCCTGCTTGCTGATATTGCAGTTTTTGTTTACAAGAATAATT 880

Consensus     AGCAATACTGAGTGAAGGATGTTGGCCAAAAGCTGCTTTCCATGGC
9-repeat      AGCAATACTGAGTGAAGGATGTTGGCCAAAAGCTGCTTTCCATGGC 886
10 DraI +     AGCAATACTGAGTGAAGGATGTTGGCCAAAAGCTGCTTTCCATGGC 926
10 DraI -     AGCAATACTGAGTGAAGGATGTTGGCCAAAAGCTGCTTTCCATGGC 926
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Figure 2 Multiple sequence alignment of human dopamine transporter 3′-untranslated regions for three different alleles reported in this study. An allele with nine tandem repeats is compared to two alleles of the same length, each having ten tandem repeats. *: A single nucleotide polymorphism at position 784 (T or C) distinguishes the two ten repeat alleles, 10 *DraI*+ and 10 *DraI* –. ▼: marks the beginning of each repeat unit.

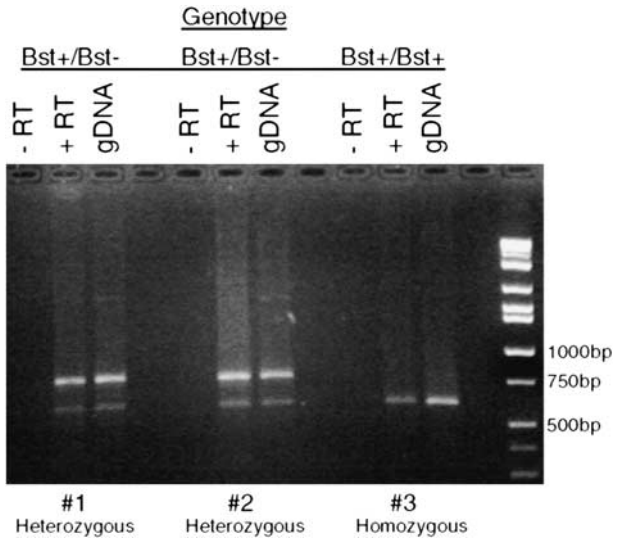

Figure 3 Both alleles (3′-UTR polymorphic forms) of the rhesus monkey dopamine transporter gene get expressed in the substantia nigra. Total RNA and genomic DNA (gDNA) were isolated from the substantia nigra of three rhesus monkeys. Total RNA was subjected to a reverse transcription reaction with (+RT) and without (–RT) reverse transcriptase. +RT, –RT and genomic DNA samples were amplified for the 3′-untranslated region of the dopamine transporter gene by PCR. Two alleles of fixed length result from a single nucleotide polymorphism and can be distinguished by restriction endonuclease (*Bst1107I*) digestion of the PCR amplification products. Identical banding patterns are observed in the +RT and gDNA lanes, indicating that both alleles are expressed in the brain. Two animals (No. 1 and No. 2) are heterozygous, having one allele that is sensitive to *Bst1107I* digestion (lower band) and one allele that is insensitive (Bst+/Bst– genotype). One animal (No. 3) is homozygous, having two alleles that are sensitive to *Bst1107I* digestion (only a lower band). The –RT lanes have no detectable bands, indicating the absence of genomic DNA contamination as well as crossover contamination in the +RT lanes.

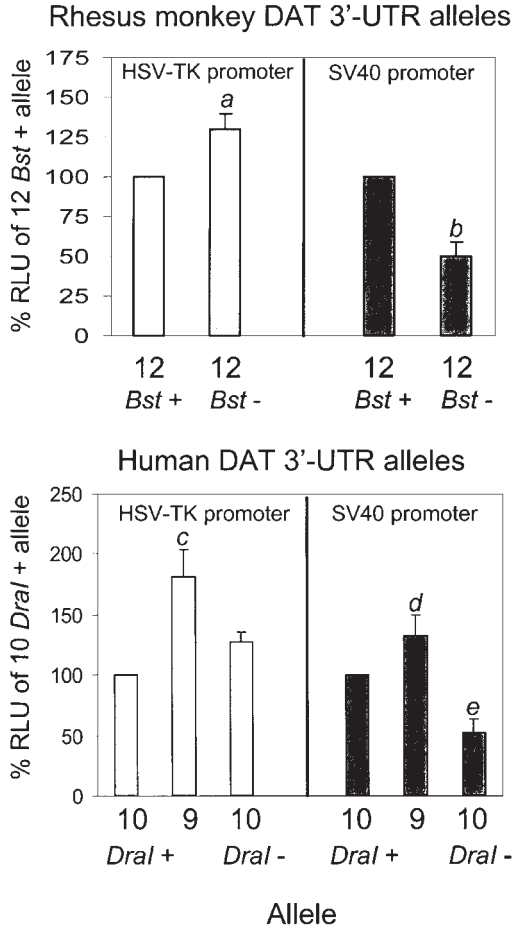

Figure 4 Alleles of the 3′-untranslated region (3′-UTR) of the human or monkey dopamine transporter have differential effects on reporter gene expression in HEK-293 cells. The 3′-UTR of different primate DAT alleles was subcloned downstream from a firefly luciferase reporter construct driven by either the herpes simplex virus thymidine kinase promoter (HSV-TK promoter) or the SV40 enhancer and early promoter (SV40 promoter). Top: Vectors containing rhesus monkey DAT 3′-UTR alleles have a fixed number of 12 tandem repeat sequences. Alleles are distinguished by the presence (Bst+) or absence (Bst–) of a single nucleotide polymorphism sensitive to the restriction enzyme *Bst1107I*. Bottom: Vectors containing human DAT 3′-UTR alleles are designated according to the number of tandem repeat units (10 or 9) and for the 10-repeat alleles, whether or not a single nucleotide polymorphism sensitive to the restriction enzyme *DraI* is present (DraI+) or absent (DraI–). Luciferase was measured as outlined in Materials and methods. Relative light units (RLU) were measured in each of three to six independent experiments (Table 1) and are expressed as percent of values derived from the 10-repeat DraI+ values (human) or the 12-repeat Bst+ values (rhesus monkey), which were considered 100%. Statistical significance was determined by ANOVA followed by Tukey's post-hoc comparisons: *a*: $P < 0.05$ *vs* 12 Bst+; *b*: $P < 0.01$ *vs* 12 Bst+; *c*: $P < 0.01$ *vs* 10 DraI+ and $P < 0.05$ *vs* 10 DraI–; *d*: $P < 0.01$ *vs* 10 DraI–; *e*: $P < 0.05$ *vs* 10 DraI+.

Luc/10-repeat-*DraI*– vector (Figure 4, *d*: $P < 0.01$, $n = 6$). In contrast with the HSV-TK promoter, however, the SV40 promoter was more sensitive to the presence of the (*DraI*) SNP. In this regard, statistically significant differences in levels of luciferase were observed in comparing pSV40-Luc/10-repeat-*DraI*– and pSV40-Luc/10-repeat-*DraI*+ (Figure 4, *e*: $P < 0.05$, $n = 3$). Data from individual experiments are summarized in Table 1.

In summary, the human 9-repeat sequence yielded higher luciferase activity than the 10-repeat sequence and this finding was irrespective of the promoter. However, a single nucleotide polymorphism in both the human and rhesus monkey DNA sequences also influenced levels of protein expression, but in a promoter-sensitive fashion.

Discussion

The present study reveals that transfection of reporter plasmids containing polymorphic forms of the human or monkey DAT 3′-UTR can result in increases or

decreases in reporter gene expression. The findings provide robust experimental evidence that variability in the length or the sequence of the 3′-UTR of the DAT gene may influence levels of DAT protein in the brain. The DAT plays a critical role in modulating dopamine transmission by regulating dopamine availability in the synapse.[3,46,47] This precept has driven intensive investigation of variability in the DAT gene that may contribute to regulation of DAT expression, function and consequent pathological states. The discovery of a variable number of tandem repeat (VNTR) sequences in the 3′-UTR of the human DAT gene[25] led to widespread screening of populations to examine the frequency of the repeat sequences.[26–28] A high level of variability in the number of repeat sequences precipitated examination of whether a particular length was associated with the incidence of neuropsychiatric disorders in which dopamine is implicated. With the exception of a small (<4%) but significant association between 10-repeat length alleles in the 3′-UTR and ADHD,[29–33] analysis of DAT alleles based on the number of repeat sequences (*length*) of the VNTR has yielded negative or inconsistent data.[41,45,48–56]

In view of mounting evidence that the 3′-UTR of genes may contribute to gene expression,[57,58] we and others extended the search for variability in primate DAT genes beyond length, to SNPs in the 3′-UTR.[22–24,44,45] We identified a 3′-UTR of a human 10-repeat DAT allele containing a novel SNP that altered a *DraI* restriction site (DraI–). On the basis of these and other findings,[22–24,44,45] we conjectured that individuals defined as 'homozygous' on the basis of the number of repeats in the VNTR may actually harbor two distinctly different alleles based on SNPs. The recent discovery of five SNPs in the 5′-flanking region of the human DAT gene in a sample of 119 unrelated Caucasians, resulting in 15 different halotypes,[59] supports this notion, as the majority of alleles of the DAT gene represented in Caucasians contain 10 repeat units in the VNTR.[26–28] Furthermore, this concept extends to other monoamine transporters, as 10 novel sequence variants in the human serotonin transporter gene (5-HTTLPR) have recently been reported.[60] For both transporter genes, alleles were initially thought to be unique on the basis of length. Thus, sequence divergences in the DAT gene may give rise to many alleles that may share a common length of the 3′-UTR.

We also identified several variable nucleotides in the 3′-UTR of the rhesus monkey DAT gene. Analysis of one that altered a *Bst1107I* restriction site in a large cohort of monkeys could distinguish separate alleles, even though the length of a repeat region in the 3′-UTR was invariant (12 repeats) in rhesus monkeys. Interestingly, the occurrence of this SNP was suggestive but not wholly predictive of levels of spontaneous activity in the rhesus monkey.[44] We concluded that the primate 3′-UTR of the DAT gene was readily differentiated either by the number of repeat units and/or by SNPs.

If particular alleles of the DAT gene differentially contribute to altered levels of DAT protein, it is important to consider both types of polymorphisms as potential modulators. Based on these considerations, we chose to investigate whether both the number of repeat sequences and the particular SNPs in the 3′-UTR of the human and nonhuman primate DAT genes that we recently cloned[44,61] may modify levels of reporter gene expression, as measured by the luciferase reporter assay. For these initial *in vitro* studies, we chose not to use a DAT cDNA as the reporter gene. Using confocal microscopy, we recently observed that DAT protein is localized to the extracellular membrane and intracellularly in transfected HEK-293 cells, and appears to redistribute and aggregate in the presence of dopamine in a time-dependent manner.[61,62] DAT function and/or density may therefore vary with DAT distribution, level of expression, media formulation or cell confluence. Other confounds arise if using post-mortem brain samples to assess whether DAT promoter-driven expression in dopamine neurons is allele dependent, since post-mortem interval, clinical history, and drug use may affect DAT density. The most direct approach would be to measure DAT density in brain using PET (or SPECT) imaging, but this approach introduces different variables, as it is not feasible to obtain full density measures and test-retest variability may exceed gene-related variance. Furthermore, it is premature to launch a costly brain imaging project, in view of the emerging gaps in our knowledge of the range of DAT polymorphisms in non-coding areas of the gene and their population frequency. In contrast, the luciferase reporter assay is a highly quantifiable and reliable methodology that has been used to examine the function of the 3′-UTR of a variety of other genes.[63–67] To control for experimental error, we prepared new DNA preps and dilutions for each experiment, tested the vectors simultaneously and repeated each experiment three to six times to confirm reproducibility (Table 1). The results were robust and statistically significant. The hypothesis, that both variable length and SNPs in the 3′-UTR of the primate DAT genes could affect reporter gene expression, was borne out and leads directly to addressing whether DAT protein levels are also affected in an analogous manner.

To assess the function of the 3′-UTR of the DAT gene, we compared the effects of several variables on luciferase gene expression, including the promoter, the length of repeat sequences and SNPs. Foremost, the number of tandem repeat sequences in the VNTR region of the human DAT 3′-UTR was an important contributor to levels of reporter gene expression. With either the HSV-TK or SV40 promoter, vectors containing the 9-repeat sequence yielded higher levels of luciferase gene expression than vectors containing either 10-repeat sequence (Table 1, Figure 4). SNPs also modified reporter gene expression. When we compared data derived from SV40-driven expression vectors that contained either a human DAT 3′-UTR segment of a *DraI*-sensitive 10-repeat allele or a *DraI*-insensitive 10-repeat allele, significant differences in the levels of luciferase were observed (Figure 4). These vectors differ by a single nucleotide. Although the frequency of the *DraI* SNP in the human population is unknown, an

SNP was also discovered in the 3′-UTR of the monkey DAT gene. In our previous study of 22 rhesus monkeys, we detected a *Bst1107I*-sensitive SNP that is very common, with about 68% of fixed-length allele PCR products sensitive to *Bst1107I* digestion. We discovered that both alleles of the DAT gene were expressed in primate brain substantia nigra, indicating the potential biological relevance of both alleles. We then examined whether this SNP could affect reporter gene expression *in vitro*. Expression vectors that contained a 3′-UTR segment of a *Bst1107I*-sensitive 12-repeat allele or a *Bst1107I*-insensitive 12-repeat allele resulted in significantly different levels of luciferase expression. In parallel to the results with the *DraI* SNP in the human DAT 3′-UTR, the influence of the rhesus monkey DAT 3′-UTR sequences depended on the promoter in the vector (Figure 4). The *Bst*-insensitive SNP (*Bst*–) increased luciferase expression with the HSV-TK promoter but reduced it with the SV40 promoter.

It is critical to consider that both the direction and the magnitude of luciferase levels that we observed in HEK-293 cells may be influenced by a complement of cell-specific proteins interacting with the 3′-UTR sequences and may not be reflective of physiological regulation of the DAT gene as it occurs in dopamine neurons. Furthermore, the luciferase assay may oversimplify and not fully reflect regulatory mechanisms underlying DAT expression in the brain. The structure and half-life of the luciferase protein and mRNA could provide an altered context that is not relevant for DAT studies. With these caveats in mind, the data suggest that the 3′-UTR of DAT alleles might interact with the SV40 or HSV-TK promoters. Furthermore, DAT 3′-UTR alleles that differ in a single nucleotide but share a common length can differentially affect reporter gene expression in HEK-293 cells.

Is it possible to relate DAT gene polymorphisms with levels of DAT protein in brain? Imaging agents that label the DAT non-invasively have enabled quantification of DAT density in living brain.[6,10,68,69] As described previously, contradictory data were reported when comparing individuals harboring 9- and/or 10-repeat length alleles.[38,43] As ADHD has a small but significant association with DAT 10-repeat length alleles, several groups investigated whether DAT levels in ADHD brains deviate from the normal range. In three SPECT studies of adults with ADHD, elevated levels of DAT protein were detected, but genotyping was not performed in this cohort.[12,13,14] As ADHD is most likely polygenic,[70–72] and the association of the 10-repeat length allele accounts for only 3.6% of the variance in hyperactive-impulsive symptoms and 1.1% of the variance in inattentive symptoms in ADHD,[31] it is unlikely that a robust association between DAT (length) genotype and DAT density would emerge. A detailed analysis of the frequency of SNPs throughout human 10-repeat length-containing alleles is needed to further investigate DAT gene polymorphisms and ADHD, as well as other dopamine-related disorders. Accordingly, the relationship between the sequence of the 3′-UTR and DAT gene expression remains unresolved.

Regardless of the allele frequency in human populations, the SNP we detected in the human DAT gene significantly affects reporter gene expression with the SV40 promoter in HEK-293 cells. Other SNPs may also influence levels of gene expression. The relevance of these observations to DAT gene regulation *in vivo* warrants a careful determination of the location and frequency of SNPs in the human DAT 3′-UTR as well as the 5′-UTR and other non-coding regions. Accordingly, a primary objective will be to clone and characterize the diversity of DAT alleles and determine whether protein levels are affected in an experimental system that most closely resembles dopamine neurons *in vivo*.

Hypothetically, polymorphisms in the 3′-UTR may either enhance or impede DAT gene expression, affect mRNA stability or alter protein recognition sites, resulting in altered DAT protein levels in the brain. With regard to the *DraI* (recognition site: TTT/AAA) polymorphism in the human DAT 3′-UTR, structural discontinuity in a TTTTAAAA sequence has been shown to alter a molecular geometry in the minor groove of DNA and nuclease accessibility to the DNA,[73] suggesting a possible functional mechanism by which the *DraI*-insensitive SNP reported in the present study (sequence: TTCTAAAA), or the *DraI*-insensitive SNP in Genbank that our cloning strategy[44] had been based on (Genbank entry AI768774; reverse compliment sequence: TTATAAAA) could alter gene expression. By analogy, the Neurofibromatosis type 1 gene (NF1) in lymphoblast cell lines harbors different NF1 alleles with polymorphisms in the 3′-UTR that lead to unequal levels of expression.[74]

Potential interactions between the promoter and the 3′-UTR were borne out by the use of two promoters in our study. The commonly used SV40 and HSV-TK promoters are the most appropriate promoters for these *in vitro* studies. They were selected because they provide low to moderate levels of expression in HEK-293 cells in contrast to other viral promoters, like CMV. Our conclusion that reporter gene expression is guided by the promoter and modified by an interaction with the 3′-UTR is also supported by similar interactions between other 3′-UTRs (SV40 early region and the rabbit β1-globin-encoding gene) and promoters (human CMV, murine CMV and SV40) across multiple cell lines.[75] Studies that compare different promoters in reporter assays are uncommon. Thus, reporter gene expression levels are dependent upon multiple factors that include the promoter in the vector, the cell line into which the vector is transfected, and the 3′-UTR. Why two different promoters yield opposite results dependent upon an SNP in the 3′-UTR remains unclear. The recent discovery of SNPs in the 5′-flanking region of the human DAT gene[59] raises the possibility that functional SNPs may exist in the promoter or other 5′ regulatory elements in the DAT gene. Our data may imply that a DAT 3′-UTR of a particular sequence might function differently depending on the sequence of the DAT promoter or other regions of the DAT gene in any given allele. The findings support the need to investigate the interaction between the native

DAT promoter and 3′-UTR of relevant polymorphisms directly or in an appropriate cell line that closely mimics dopamine neurons.

In conclusion, polymorphisms of length or of single nucleotides in the 3′-UTR of the DAT gene may contribute to the dynamic processes that regulate DAT density in the brain. Consequently, the possible grouping together of multiple alleles solely by the number of repeat sequences (*length*) of the 3-UTR, may have confounded previous investigations on the association of the DAT gene with dopamine-related disorders and masked the relevance of other sequence variations which may be involved in DAT gene regulation. An emerging concept is that polymorphisms in the noncoding regions of the DAT and other monoamine transporter and receptor genes should be investigated as contributors to the etiology of neuropsychiatric disorders, neurodegenerative diseases and the susceptibility to drug addiction. Although it is likely that many CNS disorders are polygenic in nature, detailed analysis of each gene implicated will reveal a comprehensive view of their etiology and expand the range of diagnostic and therapeutic targets for these disorders.

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