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Expression of the Dopamine Transporter Gene is Regulated by the 3′ UTR VNTR: Evidence From Brain and Lymphocytes Using Quantitative RT-PCR

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Genetic association studies provide considerable evidence that the 10-repeat allele of a variable number tandem repeat (VNTR) in the 3′-untranslated region (3′-UTR) of the dopamine transporter gene (*DAT1*) is associated with a range of psychiatric phenotypes, most notably, attention deficit hyperactivity disorder. The mechanism for this association is not yet understood, although several lines of evidence implicate variation in gene expression. In this study, we measured *DAT1* messenger RNA levels in cerebellum, temporal lobe, and lymphocytes using quantitative real-time reverse-transcription polymerase chain reaction. Relative to a set of four control housekeeping genes (β-actin, *GAPD*, ribosomal 18S, and β2-microglobulin) we observed that increased levels of *DAT1* expression were associated with the number of 10-repeat alleles. These data provide direct evidence that the VNTR, or another polymorphism in linkage disequilibrium with the VNTR, is involved in regulating expression of this gene.

KEY WORDS: dopamine transporter gene (*DAT1*); VNTR; expression; quantitative RT-PCR; TaqMan; attention deficit hyperactivity disorder

The dopamine transporter (DAT) mediates uptake of dopamine into neurons, and is a major target for various pharmacologically active stimulants such as cocaine and methylphenidate. Genetic association studies provide considerable evidence that the 10-repeat allele of a variable number tandem repeat (VNTR) in the 3′-untranslated region (3′-UTR) of the dopamine transporter gene (*DAT1*) is associated with attention deficit hyperactivity disorder (ADHD) [Cook et al., 1995; Gill et al., 1997; Waldman et al., 1998; Daly et al., 1999; Barr et al., 2001; Curran et al., 2001; Chen et al., in press], although there have been several non-replications reported [Palmer et al., 1999; Holmes et al., 2000; Todd et al., 2001]. The mechanism for this association is not yet understood, although several lines of evidence implicate variation in gene expression [Madras et al., 2002]. In vivo studies using single photon emission computed tomography (SPECT) show an increased density of striatal DAT in ADHD probands compared to controls and suggest there may be an association between the VNTR genotype and DAT density [Dougherty et al., 1999; Dresel et al., 2000; Krause et al., 2000]. In vitro studies suggest that the VNTR sequence enhances promoter activity and this may be regulated by the number of repeats [Michelhaugh et al., 2001]. In this study, we measured *DAT1* messenger RNA (mRNA) levels in cerebellum, temporal lobe, and lymphocytes and observed that increased levels of *DAT1* expression were associated with the number of 10-repeat alleles (brain: $P = 0.05$; lymphocytes: $P = 0.06$). These data provide direct evidence that the VNTR or another polymorphism in linkage disequilibrium with the VNTR is involved in regulating expression of this gene.

The VNTR polymorphism consists of a 40 bp sequence that most frequently occurs as nine or ten tandem repeat units, although 3–11 repeats are also observed. Its location within the transcribed 3′-UTR is interesting since these regions have been shown to play an important role in the regulation of transcription efficiency, mRNA stability, or mRNA sub-cellular localization [Mignone et al., 2002]. As described above, the association between the 10-repeat allele and ADHD is one of the most

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consistent findings in psychiatric genetics. As a consequence, there is currently a great deal of interest in the mechanisms that underlie such observations and whether, or not, any functional consequences can be ascribed to the VNTR or other polymorphic variants that are associated with this marker.

Our study employed quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) to compare the relative expression of *DAT1* in different genotype groups. Real-time RT-PCR uses a non-extendable 5′-fluorescently labeled probe which binds specifically between two conventional PCR primers. When intact, a quencher at the 3′ end of the probe prevents activation of the reporter fluorophore. During PCR, the 5′ exonuclease activity of *Taq* polymerase degrades the probe, separating the quencher from the reporter and thus increasing the emission intensity of the fluorescent signal. Relative expression of *DAT1* was determined by comparing the expression of *DAT1* to a set of four housekeeping control genes: β-actin, ribosomal 18S, glyceraldehyde-3-phosphate dehydrogenase (*GAPD*), and β2-microglobulin. We used the TaqMan Sequence Detection System (Applied Biosystems 3700, Foster City), which in our hands has proven to give reliable test re-test results, both within and between runs. Correlations between duplicate experiments are very high, approaching 1.00 for all the assays used.

We measured expression of *DAT1* in total mRNA derived from 16 lymphocyte samples and 20 brain samples (seven cerebellum and 13 temporal lobe). All samples were from separate individuals regardless of tissue, and were thus independent of each other. There was no significant difference in the expression levels of *DAT1* between the temporal lobe and cerebellum, so the two groups were combined in our analyses. As expected, expression levels of *DAT1* were found to be considerably higher in the brain regions than in lymphocytes. Although it has been previously reported that the dopamine transporter is expressed in peripheral blood lymphocytes [Amenta et al., 2001], it is possible that the regulation of expression is different compared to that in neurological tissue. We therefore performed the analysis on two groups, 'brain' and 'lymphocytes.'

Table I summarizes the results obtained in this study. It can be seen that there is a consistent decrease in relative expression of *DAT1* associated with the nine-repeat allele. This is seen both across tissue types and across the different control gene assays. In both tissues, there was only one sample with a 9/9 genotype, which was expected given the small sample size and low frequency of this genotype. It is interesting to note, however, that in both cases these individual samples show lower expression of DAT than the 9/10 genotype group. The relative expression levels of *DAT1* by genotype in brain and lymphocytes can bee seen graphically in Figures 1 and 2 respectively. The standard deviations calculated for the lymphocytic expression levels are much larger than for brain tissue, probably reflecting the ~1,000-fold lower expression of DAT in lymphocytes and thus an increase in both within and between sampling variability.

TABLE I. Relative Expression of DAT1 in Different Genotype Groups Against Four Housekeeping Control Gene Probes

	Brain (cerebellum and temporal lobe)					Lymphocytes				
Genotype	n	β-actin	18S	GAPDH	β2M	n	β-actin	18S	GAPDH	β2M
10/10	12	1.00 (0.88–1.14)	1.00 (0.85–1.24)	1.00 (0.89–1.12)	1.00 (0.65–1.68)	10	1.0 (0.59–1.76)	1.0 (0.61–1.75)	1.0 (0.58–1.83)	1.0 (0.54–1.90)
9/10	7	0.70 (0.58–0.86)	0.31 (0.26–0.36)	0.49 (0.44–0.54)	0.33 (0.25–0.46)	5	0.80 (0.53–1.36)	0.65 (0.42–1.26)	0.77 (0.51–1.29)	0.63 (0.31–1.37)
9/9	1	0.11 (0.09–0.14)	0.09 (0.08–0.09)	0.32 (0.29–0.34)	0.18 (0.15–0.21)	1	0.31 (0.12–0.41)	0.42 (0.26–0.59)	0.23 (0.12–0.37)	0.27 (0.10–0.63)
P value (10/10 vs. others)	—	0.16	0.02	0.08	0.02	—	0.22	0.008	0.23	0.04
Overall *P* value	—		0.05			—		0.06		

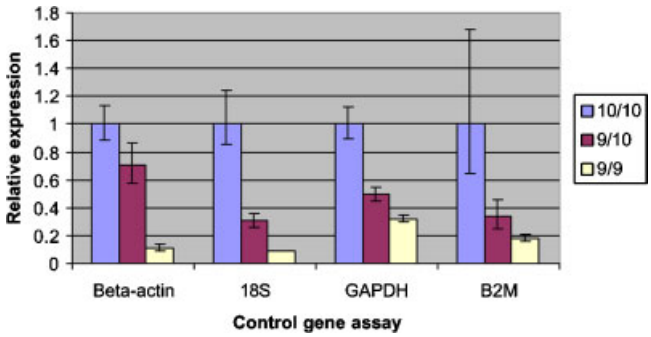

Fig. 1. DAT1 expression by genotype in brain (cerebellum and temporal lobe) (N = 20).

The results suggest that the VNTR in the 3′ UTR of the *DAT1* gene may play a role in regulating expression. It is possible that its effect is mediated through the regulation of transcription, or that it may in some way effect the efficiency of translation or the stability of mRNA. Certainly the results obtained in this study support a number of other functional studies on this polymorphism, although several ambiguities remain. As mentioned, there is good evidence to support a role of altered expression of *DAT1* in the aetiology of ADHD. Of particular interest are several studies that measured the density of DAT using SPECT in patients with ADHD and healthy controls and report an increase of ~70% in the ADHD group [Dougherty et al., 1999; Dresel et al., 2000; Krause et al., 2000]. Further evidence for a role of the dopamine transporter in ADHD is that it is the site of action for stimulant drugs like methylphenidate; SPECT studies suggest that methylphenidate lowers the increased DAT density in individuals suffering from ADHD [Krause et al., 2000]. Furthermore, the spontaneously hypertensive/hyperactive rat (SHR), considered to be one of the most valid animal models of ADHD, shows elevated levels of DAT [Watanabe et al., 1997]. Finally, *DAT1* knockout mice, which lack any expressed DAT in the brain, are a good animal model for ADHD, showing elevated activity hyperlocomotion [Giros et al., 1996].

Our data also support the hypothesis that the 10-repeat allele of *DAT1* may confer susceptibility to ADHD by altering expression of the dopamine transporter in some way. Michelhaugh et al. [2001] have demonstrated

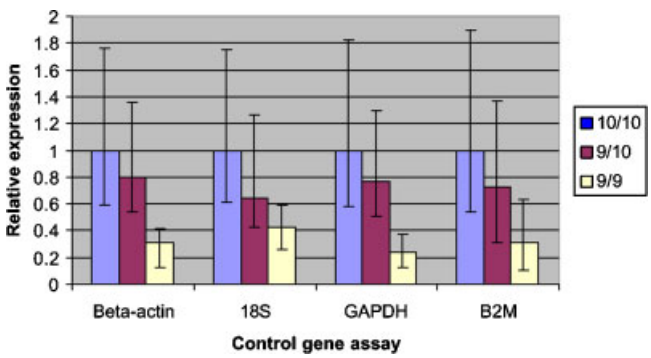

Fig. 2. DAT1 expression by genotype in lymphocytes (N = 16).

in vitro that the VNTR acts to enhance transcription of *DAT1* using green fluorescent protein (GFP) as the reporter gene. Fuke et al. [2001] used the luciferase reporter gene system and demonstrated that the 10-repeat allele increased expression compared to the nine-repeat allele in non-human primate cell line COS-7 cells; however, a more recent study using the same reporter gene system reported that the 10-repeat allele decreased expression in HEK 293 (human embryonic kidney) cells [Miller and Madras, 2002]. Furthermore, a SPECT study by Heinz et al. [2000] demonstrated increased DAT density in individuals with a 10/10 genotype compared to a 9/10 genotype, however, these findings have not been replicated in two subsequent studies [Jacobsen et al., 2000; Martinez et al., 2001]. Speculation still remains as to whether it is the VNTR itself that alters the level of expression of DAT, or if the effect is mediated by another nearby functional variant. A recent study on a number of single nucleotide polymorphisms (SNPs) within VNTR alleles of fixed length suggests that these too may alter the level of *DAT1* expression in the brain [Miller and Madras, 2002].

To conclude, our data suggest that the 10-repeat allele of a VNTR in the 3′UTR of *DAT1* enhances expression of the gene in both brain tissue and lymphocytes. This is the first study to directly measure levels of mRNA, and our results are in general agreement with data from both cell transfection and SPECT studies. The consistency of our findings between different samples, and within sample groups using different control gene assays suggests that quantitative real-time RT-PCR is an effective method for detecting differences in gene transcript expression. The fact that *DAT1* expression was detected in lymphocytes, albeit at much lower levels, and shows similar patterns to that seen in the brain supports the findings of Amenta et al. [2001]. Given the problems manifest in obtaining large quantities of well-characterized brain tissue in humans, these findings raise the possibility that lymphocytes can be used as a valuable proxy for modeling expression of the dopamine transporter. Future studies will aim to increase the number of individual samples and brain regions assayed, focussing primarily on the dopamine rich regions implicated in psychopathologies such as ADHD. We also plan to test the hypothesis that individuals with ADHD demonstrate higher levels of DAT expression than normal individuals. Following the data of Miller and Madras [2002], who found that SNPs within the 3′UTR of *DAT1* mediate expression of the gene along with the VNTR, we also plan to explore the effect of sequence variation within the repeat units of the VNTR.

MATERIALS AND METHODS

Total RNA Preparation and Reverse Transcription

Twenty brain samples were provided by the Maudsley Brain Bank (Department of Neuropathology, Institute of Psychiatry, London, UK) and stored at −70°C, prior to use. Total RNA was prepared from homogenized tissue

using Trizol reagent (Gibco BRL, UK). Blood samples were provided by 18 volunteers and total RNA was prepared from lymphocytes using QIamp RNA Blood Mini Kit (Qiagen, Crawley, UK). RNA was treated with DNase I (Qiagen) to avoid DNA contamination. The quality and purity of total RNA was assayed in a 2% agarose gel and the recovery was calculated after measuring absorbance with a spectrophotometer at 260 nm. Reverse transcription was performed using TaqMan reverse transcription reagents with random hexamers (Applied Biosystems, Foster City). All cDNA samples were tested for genomic DNA contamination by PCR amplification of a non-transcribed sequence. Samples were stored at −70°C prior to further use.

Genotyping the DAT1 VNTR

The DAT1 VNTR polymorphism was amplified on an MJ PTC-225 thermal cycler (MJ Research, Massachusetts) in a hot-start protocol involving an initial 5-min denaturing step at 95°C, followed by 38 cycles of 93°C for 1 min and 72°C for 1 min. Primers used were 5′-TGT GGT GTA GGG AAC GGC CTG AG-3′ and 5′-CTT CCT GGA GGT CAC GGC TCA AGG-3′. The reaction mix included 25 ng of cDNA, 1.5 mM $MgCl_2$, 20 mM dNTP's, 10 mM 10 × PCR Buffer (PE Applied Biosystems, Foster City), and 1 unit of *Taq* polymerase (added separately 30 s into the denaturing step). PCR products were run out on a 2% agarose gel stained with ethidium bromide.

Fluorogenic 5′ Nuclease Assay

The *DAT1* assay (primer 1: 5′-TCC TGG AAC AGC CCC AAC T-3′, primer 2: 5′-TGT GGT CCC AAA AGT GTC GTT-3′, and probe: 5′-FAM-CCC ATC CTG GTG ACT CCA GTG GAG A-3′) was designed using Primer Express (Applied Biosystems). We used four TaqMan PDARs as endogenous control gene assays: ribosomal 18S, human *GAPD*, human β-actin, and human β2-microglobulin (Applied Biosystems). Quantitative PCR was performed in triplicate for each sample on an ABI Prism 7900HT with TaqMan universal PCR master mix (Applied Biosystems) using a standard protocol. The expression data produced were analyzed and converted into threshold cycle values (Ct-values) using SDS 2.0 (Applied Biosystems). Between-run correlations for the TaqMan assays used were all high demonstrating the high reliability of these results: β-actin ($r = 0.99$); *GAPD* ($r = 1.00$); 18S ($r = 0.99$); β2-microglobulin ($r = 0.80$); *DAT1* ($r = 0.99$).

Data Analysis

Average Ct values for each sample were calculated and samples were grouped by genotype. For each assay the standard deviation was calculated. The average relative expression for each group was determined using the comparative method ($2^{-\Delta\Delta Ct}$) individually for each housekeeping control gene. Student's *t*-test and ANOVA were used to determine the significance of group differences in expression.

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