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RAPID COMMUNICATION

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A functional polymorphism in the monoamine oxidase A gene promoter

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Abstract We describe a new polymorphism upstream of the gene for monoamine oxidase A (MAOA), an important enzyme in human physiology and behavior. The polymorphism, which is located 1.2 kb upstream of the MAOA coding sequences, consists of a 30-bp repeated sequence present in 3, 3.5, 4, or 5 copies. The polymorphism is in linkage disequilibrium with other MAOA and MAOB gene markers and displays significant variations in allele frequencies across ethnic groups. The polymorphism has been shown to affect the transcriptional activity of the MAOA gene promoter by gene fusion and transfection experiments involving three different cell types. Alleles with 3.5 or 4 copies of the repeat sequence are transcribed 2–10 times more efficiently than those with 3 or 5 copies of the repeat, suggesting an optimal length for the regulatory region. This promoter region polymorphism may be useful as both a functional and an anonymous genetic marker for MAOA.

Introduction

Monoamine oxidase is a flavin-containing mitochondrial enzyme that catalyzes the degradation of several different biological amines including the neurotransmitters serotonin, norepinephrine, and dopamine. Humans and other mammals produce two monoamine oxidase isozymes with distinct substrate specificities: MAOA, which preferentially deaminates serotonin and norepinephrine, and MAOB, which acts on phenylethylamines and benzylamine (Bach et al. 1988; Donnelly and Murphy 1977; Cawthon et al. 1981; Hsu et al. 1988). Although both isozymes are found in the brain and elsewhere throughout the body, they have somewhat different cellular distributions; for example, only MAOA is present in trophoblasts, and only MAOB is present in platelets, but both isozymes are found in fibroblasts (Bond and Cundall 1977; Grimsby et al. 1990). MAOA and MAOB are encoded by two tightly linked genes that are arranged tail-to-tail on the short arm of the X chromosome between bands Xp11.23 and Xp 11.4. The cloning, physical linkage, and sequence analysis of these genes have demonstrated that they are 73% homologous and have identical intron-exon organization (Pintar et al. 1981; Kochersperger et al. 1986; Ozelius et al. 1988; Lan et al. 1989; Levy et al. 1989; Grimsby et al. 1991; Chen et al. 1992a,b).

Several lines of evidence indicate that monoamine oxidase, and in particular MAOA, plays an important role in human behavior and physiology. First, monoamine oxidase inhibitors are used to treat both depression and high blood pressure (Frazer and Hensler 1994). Second, a nonsense mutation in the MAOA gene is associated with a syndrome of mild mental retardation and impulsive aggressive behavior in affected males in a single large family studied in Holland (Brunner et al. 1993a, b). Lastly, transgenic mice with a deletion of the MAOA gene exhibit behavioral alterations, such as trembling and difficulty in righting as pups and increased aggression in adult males (Cases et al. 1995).

These observations suggest that variations in either the structural or regulatory sequences of the MAOA gene could be associated with behavioral or physiological variability in humans. Several different polymorphisms in the MAOA gene have previously been identified, including a variable number of tandem repeats (VNTR) in the first intron (Hinds et al. 1992), a dinucleotide repeat in the second intron (Konradi et al. 1992; Black et al. 1991), a G→T substitution at position 941, a T→A substitution at position 1077, and a T→C substitution at position –1460 (Brunner et al. 1993a, b). However, none of these has been shown to affect the activity or expression levels of MAOA, and attempts to demonstrate associations with behavioral traits such as bipolar disorder and alcoholism have given

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mixed results (Lim et al. 1995; Craddock et al. 1995; Vanyukov et al. 1995; Muramatsu et al. 1997). In addition, the promoter of the MAOA gene contains numerous repeated sequences (Zhu et al. 1992; Zhu and Shih 1997), but none of these has been shown to be variable. Thus, a search for additional MAOA gene polymorphisms, especially in the regulatory sequences, is warranted. We describe here a VNTR sequence located upstream of the MAOA gene (MAOA-uVNTR) and show that it affects the transcriptional potential of the MAOA promoter region.

Materials and methods

DNA samples

Genomic DNA was prepared from peripheral blood by standard methods. The subjects were participants in three NIH protocols: "Genetic Factors and Interrelationships for Cancer Risk-Related Behaviors and Complex Traits" (*n*=125), "Mapping Personality Traits to Genes" (*n*=577; Benjamin et al. 1996; Lesch et al. 1996), and "Genetic Factors and Interrelationships for Sexual Orientation, HIV Progression, Alcoholism and Psychological Traits, and Histocompatibility Antigens" (*n*=746; Hamer et al. 1993; Hu et al. 1995; Pattatucci and Hamer 1995). These protocols were approved by the appropriate NIH institutional review boards, and informed consent was obtained from all participants. For the combined sample, there were 51.5% males and 48.9% females, the average age was 33.4±13.7 years, and the ethnic composition was 76.6% White/Non-Hispanic, 3.8% Asian/Pacific Islander, 4.4% Hispanic/Latino, 3.7% African American/Black, 0.3% Native American/Alaskan, and 11.3% other or unknown.

Genotyping and DNA sequencing

Polymerase chain reaction (PCR) amplification of the MAOA-pVNTR sequence utilized two primers based on the reported sequence (Zhu et al. 1992): MAOaPT1 (5'-ACAGCCTGACCGTGGAGAAG-3', positions –1369 to –1350) and MAOaPB1 (5'-GAACGGACGCTCCATTCGGA-3', positions –1019 to –1038). Amplification was carried out in a final volume of 50 µl containing 50 ng genomic DNA, 250 µM dNTP, 10 pmol primers, and 0.5 U *Taq* DNA polymerase (GIBCO BRL) in the manufacturer's buffer with 1.5 mM $MgCl_2$. Cycling conditions were 1 min at 95°C, 1 min at 62°C, and 1 min at 72°C for 35 cycles. The PCR products were separated by electrophoresis on 1.8% Metaphor agarose gels (FMC BioProducts) and visualized by ethidium-bromide staining. The MAOA-GTn and MAOB-GTn polymorphisms were PCR amplified in the presence of ^{32}P-dCTP and analyzed by denaturing gel electrophoresis as described (Black et al. 1991; Korandi et al. 1992; Hamer et al. 1993).

For sequencing of the MAOA-uVNTR repeated region, genomic DNA samples from males with allele 1, 2, 3, or 4 of the MAOA-pVNTR were amplified as above. Reaction products were separated by agarose gel electrophoresis, purified by the NaI method (Geneclean II Kit, Bio 101), ligated to the pGEM-T Easy Vector (Promega), and cloned in *E. coli* JM109. Plasmid DNA was purified by column chromatography (Plasmid Mega Kit-5, Qiagen) and sequenced by thermal cycling (fmol DNA Cycle Sequencing System, Promega) with the MAOaPT1 and MAOaPB1 primers. For sequencing of the complete MAOA promoter region, the four pGL3 constructs described below were amplified by asymmetric PCR with three primers and sequenced by thermal cycling of the overlapping fragments. The resulting sequences were identical to one another, except for the repeat region, but differed from the published sequence (Zhu et al. 1992; GenBank accesion number m89636) at two positions: the GGG at positions –436 to –438 of the published sequence is TTT in our sequence, and the CCCCC at positions –367 to –371 in the published sequence is ACCC in our sequence.

Promoter fusion plasmid constructions

Genomic DNA samples from the same individuals as those studies for DNA sequencing were amplified by means of the following two primers: MAOa*Bgl*II (5'-GAAGATCTTCCAGAAACATGAGCACAAA-3', positions –1308 to –1289 of the reported sequence), and MAOa*Hin*dIII (5'-CCCAAGCTTGGGCTGACACGCTCCTGGGTCGTA-3', positions –6 to –28). These primers contain 10 extra bases that introduce a *Bgl*II or *Hin*dIII restriction site, repectively. Amplification was carried out in a total volume of 50 µl containing 250 ng genomic DNA, 250 µM dNTP, 10 pmol primers, and 1.75 U KlenTaq DNA Polymerase (Clontech) in the manufacturer's buffer. The samples were denatured for 5 min at 95°C, then amplified by a touchdown protocol consisting of 35 cycles of 1 min at 95°C, 1 min at 73° C (decreased by 1°C per cycle), and 1 min at 72°C, followed by a final extension for 10 min at 72°C. The resulting 1.3-kb DNA fragments containing the MAOA gene promoter and 5' untranslated sequences were purified, digested with *Bgl*II and *Hin*dIII, and ligated to *Bgl*II- and *Hin*dIII-cleaved pGL3-Basic Vector (Promega). This vector contains a modified firefly luciferase coding sequence upstream of the SV40 late poly(A) signal but no eukaryotic promoter or enhancer sequences.

Cell lines and transfection

The human neuroblastoma cell lines SY-5Y and SK-N-SH and the human placental choriocarcinoma cell line JAR were obtained from the American Type Culture Collection and grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamicin, and 2 mM L-glutamine at 37°C in 7.5% CO_2. Transfections were carried out by using a mixture of 10 µg DNA from the MAOA promoter-pGL3 firefly luciferase constructs described above together with 2 µg DNA from pRL-TK, an internal control plasmid in which *Renilla* luciferase coding sequences are fused to the *Herpes simplex* virus thymidine kinase promoter region. The column-purified plasmid DNAs were mixed with 5 µg Transfectam lipofection reagent (Promega) in 1 ml RPMI 1640 and incubated with SY-5Y cells (6.6×10^6cells/35 mm dish), SK-N-SH cells (5.6×10^5cells/35 mm dish), or JAR cells (4.5×10^5cells/35 mm dish) for 4 h at 37°C. The cells were grown for an additional 48 h, washed twice in phosphate-buffered saline, lysed in 200 µl passive lysis buffer (Promega), and briefly centrifuged to remove cellular debris. Then, 20-µl extract was sequentially assayed for firefly luciferase activity and *Renilla* luciferase activity by using Promega Luciferase Assay and Stop and Glo reagents according to the manufacturer's specifications. Luminescence measurements were performed on a Monolight 2001 luminometer with a 2-s pre-measurement delay and a 12-s measurement period. Results were expressed as the ratio of firefly to *Renilla* luciferase. All transfections were performed in triplicate, and each construct was tested in three or four independent experiments by using different plasmid DNA preparations.

Linkage and linkage disequilibrium calculations

Linkage analysis was performed by using LINKAGE 5.1 software (Lathrop et al. 1984). For linkage disequilibrium calculations, the marker alleles were grouped according to length as follows: for MAOA-uVNTR, alleles 1–2 versus alleles 3–4; for MAOA-GTn, alleles 1–6 versus 8–13; for MAOB-GTn, alleles 1–3 versus 4–9. The resulting bi-allelic systems were used to compute two measurements of the magnitude of linkage disequilibrium: R^2, the squared correlation coefficient (Sham 1998), and D^*, the proportion of maximum possible disequilibrium (Cox et al. 1998).

Results

A VNTR upstream of MAOA

The published sequence of the MAOA gene contains four exact repeats of a 30-bp sequence located at positions –1142 to –1262 relative to the ATG translation initiation codon, or approximately 1–1.2 kb upstream of the mapped transcription initiation sites (Zhu et al. 1992, 1994; Denney et al. 1994; Shih et al. 1993, 1994, 1995). This sequence is internally repetitive in that it consists of 5 repetitions of the core sequence ACC(A/G/C)G(C/T). Furthermore, the four complete repeats are followed by a half repeat consisting of the first 15 bp of the repeated motif at positions –1141 to –1127. The position and sequence of the repeated region are shown in Fig. 1.

To determine whether this repeated sequence is polymorphic, flanking primers were used to PCR-amplify genomic DNA from 98 unrelated individuals. Analysis of the reaction products by gel electrophoresis revealed several differently sized fragments, which were subcloned and sequenced. This revealed the presence of 4 variants of the MAOA upstream sequence: allele 1, which contained 3 repeats of the 30-bp sequence; allele 2, which contained 3.5 repeats; allele 3, which contained 4 repeats and was identical to the published sequence (Zhu et al. 1992; GenBank accesion number m89636); and allele 4, which contained 5 repeats. We termed this polymorphism MAOA-uVNTR (upstream variable number of tandem repeats).

Allele frequencies in different populations

The allele frequencies for MAOA-uVNTR in a total of 2156 chromosomes from four different ethnic/racial populations is shown in Table 1. Allele 1 (average frequency = 0.36) and allele 3 (average frequency = 0.62) were the most common forms in all populations studied, whereas alleles 2 and 4 were always rare. There were, however, substantial variations in allele frequency in different ethnic/racial groups. For example, allele 3 was approximately twice as common as allele 1 in the White/Non-Hispanic population, whereas allele 1 was more frequent than allele 3 in Asian/Pacific Islanders, a significant difference ($P<0.001$). The calculated heterozygosity for MAOA-uVNTR was 0.48. The observed heterozygosity for 708 females was 0.47.

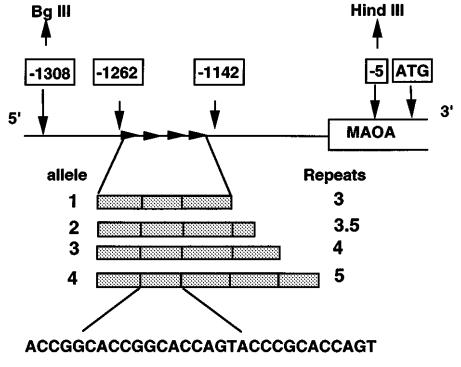

Fig. 1 The MAOA gene and the MAOA-uVNTR repeat sequence. *Numbers in boxes* indicate the positions of the MAOA-uVNTR repeat and of the sites that were used for cloning into the promoter fusion vector. The allele numbering, repeat structure, and sequence of the repeated region are also shown

Table 1 MAOA-uVNTR allele frequencies

	MAOA-uVNTR allele			
Population	1	2	3	4
Total				
n	768	12	1345	31
%	35.6	0.4	62.4	1.4
White/Non-Hispanic				
n	539	8	1056	26
%	33.1	0.5	64.8	1.6
Asian/Pacific Islander				
n	50	1	31	–
%	61.0	1.2	37.8	–
Hispanic/Latino				
n	27	–	65	–
%	29.3	–	70.7	–
African Amer/Black				
n	52	2	32	2
%	59.1	2.3	36.4	2.3

Linkage and linkage disequilibrium

Linkage and linkage disequilibrium analyses of MAOA-uVNTR were conducted by using two additional markers from the MAO gene cluster: MAOA-GTn, a dinucleotide repeat located in the second intron of the MAOA gene (Black et al. 1991), and MAOB-GTn, a dinucleotide repeat located in the second intron of the MAO-B gene (Korandi et al. 1992; Grimsby et al. 1992) As expected, the MAOA-uVNTR displayed strict X-linked inheritance in families. Linkage analysis revealed no obligatory recombinants between any of the markers, giving linkage statistics of LOD=28.2 at $\Phi=0$ for MAOA-uVNTR/MAOA-GTn (276 potentially informative meioses) and LOD=15.8 at $\Phi=0$ for MAOA-uVNTR/MAOB-GTn (134 potentially informative meioses).

Linkage disequilibrium was assessed by examining X-chromosome haplotypes in 635 male subjects. The haplotypes for MAOA-uVNTR/MAOA-GTn and for MAOA-uVNTR/MAOB-GTn are cross-tabulated in Tables 2 and 3, respectively. There was strong disequilibrium between the MAOA-uVNTR and MAOA-GTn markers, which are located just 24 kb apart; the shorter MAOA-GTn alleles were found most frequently on chromosomes carrying MAOA-uVNTR allele 1, whereas the longer MAOA-GTn alleles appeared most frequently on chromo-

Table 2 MAOA-uVNTR/MAOA-GTn haplotype frequencies

MAOA-GTn allele		MAOA-uVNTR allele 1	2	3	4
1	*n*	1	–	–	–
	%	0.2	–	–	–
2	*n*	1	–	–	–
	%	0.2	–	–	–
3	*n*	32	–	–	–
	%	5.0	–	–	–
4	*n*	5	–	–	–
	%	0.8	–	–	–
5	*n*	119	–	–	–
	%	18.7	–	–	–
6	*n*	10	–	2	–
	%	1.6	–	0.3	–
7	*n*	8	–	6	–
	%	1.3	–	0.9	–
8	*n*	25	–	81	2
	%	3.9	–	12.8	3
9	*n*	11	–	295	5
	%	1.7	–	46.5	0.8
10	*n*	2	5	17	–
	%	0.3	0.8	2.7	–
11	*n*	–	–	3	2
	%	–	–	0.5	0.3
12	*n*	1	–	–	–
	%	0.2	–	–	–
13	*n*	2	–	–	–
	%	0.3	–	–	–

Chi-square=651, *df*=36, *P*<0.00001
*D**=0.98, *P*<0.00001
R^2=0.47, *P*<0.00001

Table 3 MAOA-uVNTR/MAOB-GTn haplotype frequencies

MAOB-GTn allele		MAOA-uVNTR allele 1	2	3	4
1	*n*	3	–	10	–
	%	0.7	–	2.4	–
2	*n*	43	–	98	2
	%	10.2	–	23.2	0.5
3	*n*	46	4	31	–
	%	10.9	0.9	7.3	–
4	*n*	21	–	75	3
	%	5.0	–	17.8	0.7
5	*n*	19	–	38	–
	%	4.5	–	9.0	–
6	*n*	–	–	23	–
	%	–	–	5.5	–
7	*n*	–	–	5	–
	%	–	–	1.2	–
9	*n*	1	–	–	–
	%	0.2	–	–	–

Chi-square=69, *df*=21, *P*<0.00001
*D**=0.14, *P*=0.0007
R^2=0.05, *P*=0.0007

somes carrying MAOA-uVNTR allele 3. After grouping the marker alleles according to length, the calculated linkage disequilibrium statistics were *D**=0.98 for the percentage of maximum possible disequilibrium, and R^2=0.47 for the squared correlation coefficient. There was also significant linkage disequilibrium between MAOA-uVNTR and MAOB-GTn, which are 110 kb apart, but the extent of association was less than for the MAOA marker; *D**=0.14 and R^2=0.05.

Effect on promoter function

The location of the MAOA-uVNTR suggested that it might have an effect on transcription from the MAOA promoter. To test this, promoter DNA fragments containing the four different MAOA-uVNTR alleles were fused to a firefly luciferase reporter gene in a promoterless vector (pGL3-basic vector). The MAOA gene fragments extended from position –1308 (46 bp upstream of the repeated sequence) to position –6 (in the MAOA 5'-untranslated region) and thus contained all of the identified MAOA transcriptional regulatory and initiation sequences (Zhu et al.1992; Denney et al.1994; Denney 1995). DNA sequencing of the complete MAOA promoter fragments showed that they were identical except for the repeat region. The constructs were co-transfected with a *Renilla* luciferase gene plasmid as an internal control into three different cultured cell lines: SY-5Y and SK-N-SH, which are derived from human neuroblastomas, and JAR, a human placental choriocarcinoma cell line. The activity of the MAOA promoter fusion fragments was determined 48 h post-transfection by measuring the ratio of firefly to *Renilla* luciferase luminescence.

The MAOA-uVNTR polymorphism had a significant effect on reporter gene transcription (Fig. 2). In each of the cell lines tested, constructs containing alleles 2 and 3 were expressed at higher levels than constructs containing alleles 1 and 4. The observed ratios of transcription ranged from a maximum of 9.6-fold for allele 2/allele 4 in JAR cells to a minimum of 2.4-fold for allele 2/allele 1 in SY5Y cells. For the two most frequent alleles in the population, alleles 1 and 3, the ratio of reporter gene transcription was allele 3/allele 1=4.8 in JAR cells, 2.7 in SK-N-SH cells, and 2.8 in SY5Y cells. The differences between constructs were statistically significant in repeated experiments with different plasmid DNA preparations. Constructs lacking the entire repeat region (i.e., a fragment extending from position –1083 to position –6) gave reporter gene expression levels similar to constructs containing allele 1 or allele 4 (data not shown). Thus, MAOA-uVNTR alleles 2 and 3 appear to act as upstream activators of transcription, whereas alleles 1 and 4 do not have this ability.

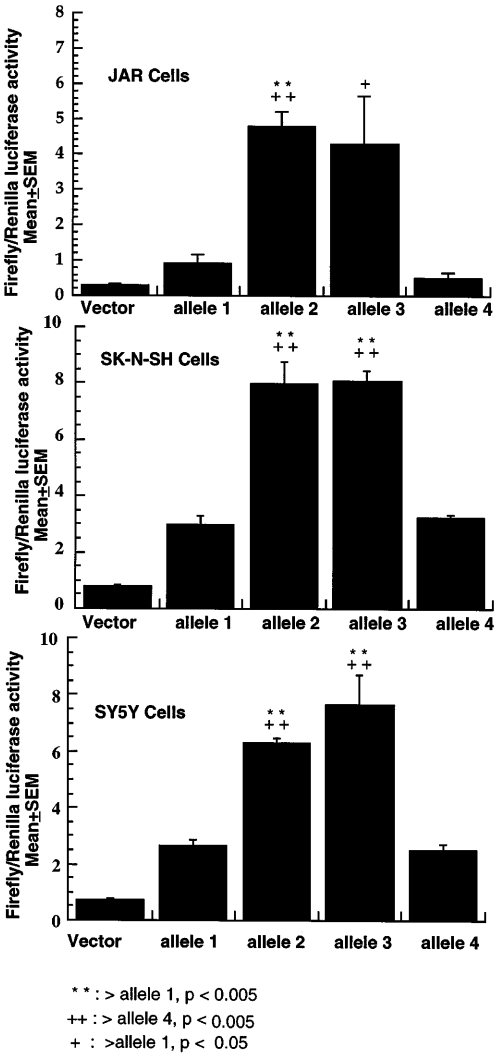

Fig. 2 Promoter fusion assays. The indicated cell lines were transfected with constructs in which firefly luciferase was fused to the MAOA promoter carrying allele 1, 2, 3, or 4 of the MAOA-uVNTR or with the vector alone. The cells were co-transfected with a control plasmid containing *Renilla* luciferase. The ratios of firefly to *Renilla* luciferase (mean±SEM) are shown for one out of three to four similar independent experiments in which each plasmid was assayed in triplicate. The observed levels of luciferase (firefly/*Renilla*, in arbitrary luminescence units) were as follows. JAR cells: allele 1, 529/575; allele 2, 3231/757; allele 3, 2913/735; allele 4, 495/816. SK-N-SH cells: allele 1, 7189/1985; allele 2, 18722/2200; allele 3, 16153/2003; allele 4, 6704/1919. SY5Y cells: allele 1, 4475/1414; allele 2, 9060/1331; allele 3, 10945/1284; allele 4, 4552/1427

Discussion

We have described a common polymorphism that lies in the promoter region of the MAOA gene and that may serve as a functional and anonymous marker of MAOA gene function. The evidence that the MAOA-uVNTR influences transcription from the MAOA gene promoter comes from gene fusion and transfection experiments. In each of three different cell lines, alleles containing 3.5 or 4 repeats of the 30-bp sequence are expressed significantly more efficiently than alleles containing either 1 or 4 repeat sequences. This suggests that there is an optimal length for the repeat region to act as a transcriptional activator element. It is not yet known whether the uVNTR influences MAOA gene expression by serving as a binding site for a transcription factor or by altering the chromatin structure of the gene, nor is it known whether the effects observed with cloned fusion genes in transfected cells are reflective of the chromosomal gene in its natural setting.

Recently, a polymorphic repeated sequence lying 1 kb upstream of the serotonin transporter gene has been shown to influence transcription of the endogenous gene in lymphoblasts (Lesch et al. 1996) and in brain (Little et al. 1998) in a manner similar to that observed for the cloned gene in transfection experiments. This polymorphism has been associated with anxiety-related personality traits (Lesch et al. 1996) and with affective disorders (Collier et al. 1996). Similarly, the insulin gene contains a 5' flanking VNTR that has been associated with insulin-dependent diabetes mellitus (Bennett et al. 1995; Pugliese et al. 1997) and that controls transcription in vivo through the transcription factor Pur-1 (Kennedy et al. 1995). Given that polymorphic sequences such as VNTRs are far more common in control regions than in coding sequences, perhaps they play a frequent role in complex multigenic traits.

Previous attempts to demonstrate associations of the dinucleotide repeat in the second intron of the MAOA gene with common behavioral conditions have given variable results (Lim et al. 1995; Craddock et al. 1995; Vanyukov et al. 1995; Muramatsu et al. 1997). We have shown that the dinucleotide polymorphism is in strong linkage disequilbrium with MAOA-uVNTR, suggesting that it might be fruitful to reinvestigate these associations with more complete genotype information; i.e., MAOA-uVNTR/MAOA-GTn haplotypes. Because we have also found significant ethnic/racial variations in MAOA-uVNTR allele frequencies, it will be important to control for population stratification in such studies by careful ethnic matching or by within-family designs. It would also make sense to analyze the results in terms of the transcriptional efficiency of the different alleles rather than their length. In a preliminary analysis, we have found a trend toward association of high transcribing alleles of MAO-uVNTRn with self-reported depression and low mood; it will be interesting to determine whether this holds true in additional studies, especially of populations with diagnosed mood disorders.

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