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ORIGINAL RESEARCH ARTICLE

Family-based and association studies of monoamine oxidase A and attention deficit hyperactivity disorder (ADHD): preferential transmission of the long promoter-region repeat and its association with impaired performance on a continuous performance test (TOVA)

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Monoamine oxidase A (MAO A) is located on the X chromosome and metabolizes biogenic amines including dopamine, norepinephrine and serotonin. A functional promoter-region polymorphism of this gene has been described that has been studied in a number of mental illnesses but not in attention deficit hyperactivity disorder (ADHD). In the current study, we examined the MAO A promoter-region polymorphism initially in 133 triads and observed preferential transmission of the long alleles from 74 heterozygote mothers to ADHD probands (χ^2 = 4.37, *P* = 0.036, df = 1). We also examined the role of this polymorphism in a computerized continuous performance test, the TOVA. Significant differences were observed on errors of commission (χ^2 = 7.021, *P* = 0.008) and patients carrying the long MAO A allele made significantly more such errors. Errors of commission are a measure of impulsivity. However, following Ritalin (methylphenidate) administration the association between this polymorphism and errors of commission was markedly attenuated and no longer significant at the *P* < 0.05 level. We also analyzed the provisional association by the case-control design. A significant difference in allele frequency was observed between 110 male probands *vs* 202 male controls (Pearson χ^2 = 7.94, *P* = 0.047). Similarly results were obtained when 19 female probands were compared to female controls (genotype χ^2 = 21.28; *P* = 0.0032, 3 df and allele χ^2 = 30.88, *P* = 0.0007, 2 df). All three complementary approaches employed (family-based, case-control and quantitative trait design) suggest a role for the MAO A promoter-region polymorphism in conferring risk for ADHD in our patient population.
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A gene of potential interest in ADHD, since it metabolizes biogenic amines and is located on the X chromosome, is monoamine oxidase A (MAO A). X chromosome genes are worthy candidates for studies in this disorder as ADHD is relatively infrequent among girls.[1,2] Two related genes, MAO A and MAO B likely arising from duplication of an ancestral gene, map to Xp11.23. MAOA and MAOB genes span at least 60 kb, consist of 15 exons, and exhibit identical exon–intron organization.[3] This enzyme plays a central role in metabolism of amine neurotransmitters.[4] MAO A preferentially oxidizes serotonin (5-hydroxytryptamine, 5-HT) and norepinephrine (NE), whereas MAO B preferentially oxidizes phenylethylamine. Both forms can oxidize dopamine (DA). However, the substrate specificity overlap and the *in vivo* function of these two isoenzymes remain to be clarified.

MAO A is likely to play an important role in human behavior for several reasons. Firstly, MAO A inhibitors are effective antidepressants.[5,6] Secondly, Brunner and his colleagues have described a nonsense mutation in the MAO A gene that is associated with mild mental retardation and impulsive aggressive behavior in affected males in a single large Dutch family.[7] Finally, transgenic mice lacking the MAO A gene are characterized by aggressive behavior and higher brain levels of monoaminergic neurotransmitters.[8]

A number of polymorphic sites have been described

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for these two enzymes,[9,10] including a dinucleotide repeat in MAO A,[11,12] but the most noteworthy is a repeat region in the MAO A promoter region.[13] This polymorphism is located 1.2 kb upstream of the MAOA coding sequences, consists of a 30-bp repeated sequence present in 2 (291 bp), 3 (321 bp), 3.5 (336 bp), 4 (351 bp), or 5 (381 bp) copies. Expression studies[13–16] show that the number of repeats is related to the transcriptional efficiency of the gene. The promoter region repeat has now been examined for possible association with mood disorder,[16–20] personality,[18] impulsivity-aggressivity,[21] alcoholism[22,23] and panic disorder.[14,24]

Payton and his colleagues analyzed a number of candidate genes in the DA pathway[25] in ADHD families including the MAO A dinucleotide repeat[11] and found a trend for the preferential transmission of the 122 bp allele. Jiang *et al*[26] reported linkage between the DXS7 microsatellite, which is closely linked to the two MAO genes, and ADHD. These two reports suggest that these markers may be in linkage disequilibrium with a third, functional polymorphism.

Towards clarifying the role of the MAO A gene in ADHD, we employed three complementary approaches. We tested for linkage between the MAO A promoter region polymorphism and ADHD by using the transmission disequilibrium test[27,28] in a group of 133 triads (proband and both parents). We also employed a case-control approach and tested for association between 110 male ADHD probands compared to 202 control subjects and between 19 female ADHD probands compared to 265 female control subjects. Lastly, we evaluated the effect of the MAO A polymorphism on a quantitative trait known to be impaired in ADHD,[29] by testing 112 subjects before and after Ritalin treatment using a computerized continuous performance test—the **T**est **O**f **V**ariables of **A**ttention or TOVA.[30] There is a growing consensus that the increased penetrance of quantitative traits[31–34] and their closer relation to the gene than that of the phenotype proper may be a valuable complement to linkage[35] and association studies in psychiatric genetics.

Methods and materials

ADHD cases and parents were recruited from the greater Tel-Aviv (Petak Tikvah) municipal area ($n = 133$ families). Subjects were all clinical referrals from hospital neurologists, school psychologists and parents.

Families included all diagnosed ADHD children who had two biological parents. The Ethics Committee of the Israeli Health Ministry approved this study and written informed consent was obtained from participating subjects. ADHD criteria followed DSM-IV guidelines that recognize three types of ADHD: ADHD-Predominantly Inattentive (Type I), ADHD-Predominantly Hyperactive Impulsive (Type II), and ADHD-Combined Type (III). Informants were the parents, the teacher, and the proband. The parents and the proband underwent a thorough, albeit not standardized, clinical interview, which included as a separate item all DSM-IV criteria for ADHD and Conduct Disorder. Two scales, the *abbreviated* Conners Rating Scales[36] and the Child Behavior Checklist,[37] were also employed. Consensus diagnoses were made according to DSM-IV ADHD or either with or without comorbidity. These DSM-IV diagnoses were based on all available clinical information and the Child Behaviour Checklist and the Conners Parents and Teachers Rating Scales. When the Conners teachers scale was not available, teachers were contacted by telephone and interviewed.

The ADHD subjects were also administered the **T**est **O**f **V**ariables of **A**ttention (TOVA), which is a computerized Continuous Performance Test.[30] The TOVA is a 23-minute fixed-interval visual Continuous Performance Test with minimal language demands and no left-right discrimination. The target is presented on 22.5% and 77.5% of the trials during the first and second halves, respectively. TOVA indices include omission and commission errors, response time means and standard deviations, and anticipatory responses. In the Ritalin phase subjects receive 0.3 mg kg^{-1} of drug. The computerized tests ask the subjects to press a microswitch/scorebox with a ± 1-millisecond variance whenever the appropriate 'target' or stimulus appears on the screen. The 'target' is when a little square appears in the upper portion of another square and the 'non-target' is when the little square is in the bottom portion of the bigger square. So, every 2 seconds, a stimulus will flash on the screen and the subject then responds to the 'targets' and not to the 'non-targets'. Errors of omission (inattention), errors of commission (impulsivity), response times, and other variables are all recorded for each 5-min quarter and 10-min halves, as well as overall total scores for each variable. Scores are then compared to standardized norms and an interpretation of the data is reported in a printable report.

The TOVA was the sole neuropsychological test administered to these subjects. We excluded cases with a primary diagnosis of Pervasive Developmental Disorder, physical handicap, psychosis, mental retardation, epilepsy, hyperthyroidism, evidence or history of child abuse, adoption. Children with an IQ less than 80 were excluded. IQ was assessed using the Wechsler Intelligence Scale for Children- Revised.[38]

The probands consisted of 86% males and 14% females from 133 families. The average age was 10.68 years ± 3.70 SD. The percentage of probands with ADHD combined type was 68.2%, inattentive 30.6% and 1.2% impulsive. Twenty-seven per cent of the probands had comorbid ODD/CD.

Genotyping

DNA was extracted from frozen blood samples using the phenol procedure or from fresh blood using a MasterPure kit (Epicentre Technologies, Madison, WI, USA). From some individuals, from whom blood sample could not be obtained, DNA was obtained from buccal smears again using the MasterPure kit.

The promoter region polymorphism was characterized using a PCR procedure with the following primers:[13] MAO P1 5′-ACA GCC TGA CCG TGG AGA

AG -3′ and MAO P2 5′-GAA CGG ACG CTC CAT TCG GA –3′. The reaction mixture (20 μl) contained 200 μM dNTPs, 0.25 μM primers, 0.5 unit TAQ Gold (PerkinElmer Life Sciences, Boston, MA, USA), and 30 ng DNA. The amplification procedure included a 12-min prestart at 95°C (for the hot start) and cycling conditions as follows: 95°C for 35 s, 64°C for 35 s and 72°C for 50 s. A final extension at 72°C for 5 min was employed. Five variants of a 30-bp repeat sequence have been reported: 2 (291 bp), 3 (321 bp), 4 (351 bp), 5 (381 bp) and 3.5 (336 bp).

Statistical analysis

The transmission disequilibrium test[27,28] was used to analyze transmission from heterozygote mothers to ADHD probands. Since Deckert and his colleagues,[14] and replicated by Sayagailo *et al*,[16] showed that the 3.5, 4 and 5 alleles are more active than the 3 alleles, subjects were grouped into two genotype classes, 2 (we observed only one case) & 3 repeats (short) and 4 & 5 repeats (long). In our analysis therefore the 2 and 3 alleles were pooled as 'short' and the 4 and 5 alleles as 'long'. The rare occurrence of the 2 and 5 alleles made superfluous the use of the ETDT statistic.[39]

The Kruskal–Wallis one-way ANOVA is the distribution-free (or nonparametric) analogue of the parametric ANOVA and was used to group TOVA scores by genotype.

Results

Genotype and allele frequency of the probands' mothers is shown in Table 1. As observed in previous studies, the most common alleles are the 3 and 4 repeats that account for 96% of the alleles. The 2 and 5 alleles are relatively rare.

Possible preferential transmission of the MAO A promoter region polymorphism from heterozygote mothers to the proband was examined using the TDT design. The distribution of transmitted alleles is shown in Table 2 and there is significant transmission of the long (4 & 5) alleles ($\chi^2 = 4.37$, $P = 0.036$, 1 df). Similar results were obtained if only transmission of the common 3 and 4 repeat alleles (excluding the 5 repeat) was considered ($\chi^2 = 2.88$, $P = 0.09$, 1 df). In our sample only the common 3 and 4 alleles, or by combining the 4 & 5 repeat as suggested by expression studies,[14,16] provided enough information to calculate the TDT statistic.

We next examined the relationship between MAO A alleles and scores on the **T**est **O**f **V**ariables of **A**ttention (TOVA), a computerized Continuous Performance Test widely used with ADHD patients.[30] As shown in Table 3, significant differences were observed on errors of commission (a measure of impulsivity), and probands with the long MAO A alleles made significantly more commission errors. Similar results were obtained when the various allelic repeats were separately examined

Table 1 Genotype and allele frequencies of the MAO A polymorphism in mothers of ADHD probands

Genotype			*Allele*		
Repeat	*Frequency*	*%*	*Repeat*	*Frequency*	*%*
24	1	0.38	2	1	0.38
33	17	6.39	3	106	39.84
34	64	24.06	4	149	56.01
35	8	3.01	5	10	3.75
44	41	15.41			
45	2	0.75			
Total	133			266	

Table 2 Count of alleles in informative triads for MAO A promoter region repeat polymorphism

Allele	*Transmitted*	*Non-transmitted*
2 allele	1	0
3 allele	27	41
4 allele	41	27
5 allele	5	4
Short (2,3)	28	46
Long (4,5)	46	28

Although there were 75 informative mothers, reliable genotyping was obtained from 74 probands.

Table 3 Kruskal–Wallis test TOVA scores grouped by MAO A allele in male ADHD subjects

Ranks	*MAO A*	*n*	*Mean rank*	*Chi-square*	*P value*
TOVA total score	3	35	56.61		
	4	73	53.49		
	Total	108		0.237	0.626
TOVA O 1 omissions	3	34	52.18		
(First half)	4	72	54.13		
	Total	106		0.095	0.758
TOVA O 2 omissions	3	34	48.81		
(Second half)	4	72	55.72		
	Total	106		1.169	0.280
TOVA O Total	3	34	49.40		
	4	73	56.14		
	Total	107			
TOVA C 1 commission	3	34	41.13		
(First half)	4	72	59.34		
	Total	106		8.202	0.004
TOVA C 2 commission	3	34	45.10		
(Second half)	4	72	57.47		
	Total	106		3.735	0.053
TOVA C Total	3	34	42.35		
	4	73	59.42		
				7.021	0.008

Table 4 Kruskal–Wallis test TOVA scores grouped by MAO A allele in male ADHD subjects following Ritalin administration

Ranks	*MAO A*	*n*	*Mean rank*	*Chi-square*	*P value*
TOVA + Ritalin	3.00	36	59.89		
	4.00	76	54.89		
	Total	112		1.877	0.171
TO′ Omissions 1	3.00	38	57.72		
	4.00	73	55.10		
	Total	111		0.190	0.663
TO′ O 2	3.00	38	54.24		
	4.00	73	56.92		
	Total	111		0.179	0.672
TO′ O Total	3.00	38	54.20		
	4.00	73	56.94		
	Total	111		0.186	0.666
TO′ Commissions 1	3.00	38	48.20		
	4.00	73	60.06		
	Total	111		3.452	0.063
TO′ C 2	3.00	38	50.36		
	4.00	73	58.94		
	Total	111		1.778	0.182
TO′ C Total	3.00	38	48.86		
	4.00	73	59.72		
	Total	111		2.849	0.091

(TOVA **C**ommission 1 $\chi^2 = 8.87$, $P = 0.012$; TOVA C2 $\chi^2 = 5.177$, $P = 0.075$l; TOVA C total $\chi^2 = 8.739$, $P = 0.013$). We next analyzed the TOVA scores following treatment with Ritalin (Table 4). In contrast to the results obtained in the absence of Ritalin, in the presence of drug only a weak ($P>0.05$) non-significant effect of the MAO A allele on test performance was observed. There was no significant effect of the MAO A alleles on reaction time in the presence or absence of Ritalin (data not shown).

We also analyzed the putative association between the MAO A promoter region polymorphism and ADHD using the case-control design. Table 5 shows the allele frequency of this polymorphism in 110 male probands and 202 non-related male control subjects recruited in our studies of normal personality.[40,41] A significant difference in allele frequency was observed (Pearson $\chi^2 = 7.94$, $P = 0.047$, df = 3). Similar results were obtained with the smaller group of female probands compared to a group of female controls (Table 6; genotype $\chi^2 = 21.28$; $P = 0.0032$, 3 df and Table 7 allele $\chi^2 = 30.88$, $P = 0.0007$, 2 df).

Table 5 MAO A polymorphism in male ADHD patients compared to male control subjects

Diagnosis		*MAO promoter region allele repeat*				*Total*
		2	*3*	*4*	*5*	
Control	Count		75	127		202
	%		37.13	62.87		
ADHD	Count	1	35	71	3	110
	%	0.90	31.82	64.55	2.73	
	Count	1	110	198	3	312

Table 6 MAO A genotype frequency in female ADHD probands *vs* control females

		MAO promoter region genotype					*Total*
		33	*34*	*35*	*44*	*45*	
Control	Count	51	120		93	1	265
	%	19.25	45.28		35.09	0.38	100
ADHD	Count	1	7	2	9		19
	%	5.26	36.84	10.53	47.37		100
	Count	52	127	2	102	1	284

Additionally, we examined if there was any relationship between MAO A genotype and scores on any of the eight subscales of the Childhood Behavioral Check List parents and teachers scales. No significant relationship (Kruskal–Wallis test, all P values >0.25) was detected between any subscales and MAO A genotype (short *vs* long alleles) for either parents or teachers (data not shown).

Discussion

We have used three complementary strategies to examine the role of the MAO A promoter region polymorphism in ADHD: (1) a family-based TDT design to test for preferential transmission of alleles to ADHD children; (2) a case-control design comparing genotype and allele frequencies between probands and non-related controls; and (3) a QTL (quantitative trait loci) design for evaluating the effect of the MAO A polymorphism on a continuous performance test. All three approaches support a provisional role for the MAO A high enzyme alleles in conferring risk for ADHD.

The TDT family-based design[27,28] is robust to population stratification and has become popular since it avoids a pitfall sometimes encountered in association

Table 7 MAO A allele frequency in female ADHD probands *vs* control females

		MAO promoter region allele repeat			*Total*
		3	*4*	*5*	
Control	Count	222	307	1	530
	%	41.89	57.92	0.19	100
ADHD	Count	11	25	2	38
	%	28.95	65.79	5.26	100
	Count	233	332	3	568

studies. However, since the application of this design is dependent on the availability of a heterozygote parent, not all families are informative. The loss of power in the TDT design is compounded when a candidate gene is on the X chromosome, as is MAO A, and only transmission from heterozygote mothers provides useful information. In the current study only 73 families out of a total of 133 were informative. Nevertheless, preferential transmission of the high enzyme activity allele was observed. Loss of information in employing the TDT design suggested that the additional use of a case-control approach would be sensible. As expected, a significant difference in MAO A allele and genotype frequencies was observed when both male and female ADHD probands were separately compared to sex-matched control subjects recruited in our studies of normal personality.[40,41] To conclude, both the family and case-control designs support a role for the MAO A gene in ADHD.

In the third approach, we studied the role of the MAO A polymorphism in partially determining a quantitative trait that is deteriorated in attention deficit.[29] Children with the long MAO A promoter region alleles (4 & 5) showed impaired performance on the TOVA test and committed significantly more errors of commission than probands with the 3 repeat. Intriguingly, the effect of the long MAO promoter region polymorphism on TOVA performance was no longer significant following Ritalin administration to the test subjects. In a sense, Ritalin neutralized and masked the effect of the MAO A gene on test performance and in the presence of the drug there was only a weak effect of allele on errors of commission. Errors of commission (the test subjects 'jumps the gun' and prematurely responds to the stimulus) are a measure of impulsivity, a principal diagnostic and clinical attribute of ADHD. It is interesting to note that the nucleus accumbens, a DA rich nucleus in the basal ganglia, has recently been implicated in mediating impulsive behavior in rats.[42]

A prime reason for implication of DA in ADHD is the evidence demonstrating that the effects of psychostimulants such as methylphenidate, that are clinically efficacious in this disorder, are mediated by DA pathways.[43] Imaging studies also show a role for DA in mediating methylphenidate effects in humans. For example, oral methylphenidate at doses within the therapeutic range significantly increases extracellular DA in human brain.[44] Excess of the MAO A high enzyme activity polymorphism in ADHD probands, would also be expected *prima facie* to decrease brain DA levels (and other monoaminergic neurotransmitters such as NE and 5-HT). Consistent with this conjecture are the the results of Jonsson *et al*[45] who examined the *in vivo* role of the MAO A functional polymorphism in humans. They found that women, carrying at least one copy of the alleles associated with more efficient transcription, displayed higher concentrations of the DA metabolite, HVA, and the 5-HT metabolite, 5-HIAA, indicating increased turnover of these neurotransmitters in the presence of the high enzyme activity allele.

Several lines of evidence support the notion that ADHD patients have reduced DA activity. (i) One of the more robust findings in molecular genetic studies of psychiatric disorders is the association between the 7 repeat allele of the DRD4 receptor and ADHD.[46] Although the neurochemical explanation of this effect needs further elucidation, *in vitro* expression studies have shown that the 7 repeat allele is somewhat *less* effective in inhibiting cyclic AMP accumulation (cyclic AMP is the second messenger mediating the DA D2 receptor class's inhibitory action on neurotransmission) than the 4 repeat.[47] (ii) It has been shown by SPECT scan that ADHD patients have increased striatal DA transporter levels that in addition would be predicted to reduce DA levels.[48] (iii) Some genetic studies support a role for the DA transporter (DAT) in ADHD[49] mediated perhaps by the *DAT1* polymorphism that may be more efficient at reuptake. (iv) High midbrain DOPA (a DA precursor) accumulation has been reported in ADHD children.[50] These considerations support the idea that decreased brain DA levels or a 'DA deficit', as proposed by Swanson,[49,51] is characteristic of this disorder and confers risk for ADHD. Presence of the long MAO A alleles would likely exacerbate DA deficiency in ADHD and add to disease risk.

In addition to the role played by DA, stimulant-induced effects on both NE[52] and 5-HT[53] have also been implicated and some evidence suggests possible roles for both these transmitters in the therapeutic efficacy of psychostimulants.[54] Consistent with a serotonergic involvement in ADHD are two recent genetic studies.[55,56] One study showed that the long/long 5-HT transporter promoter region polymorphism is overrepresented in hyperkinetic children[55] and the second study from our laboratory showed that this same genotype is overrepresented in ADHD.[56] Animal models of ADHD also support a role for both 5-HT and DA.[57,58] Since MAO A metabolizes 5-HT as well as DA, it was not possible in the current study to distinguish between serotonergic and dopaminergic pathways in mediating the effects of MAO A in ADHD or on the continuous performance test. Both DA and 5-HT may be playing a role in these effects.

The term endophenotype was used by Gottesman[59] to describe a trait that may be intermediate on the chain of causality from genes to diseases. Some family relatives of affected patients also carry the endophenotype, although not the disease phenotype. This increased penetrance of the endophenotype compared to the phenotype proper is expected to help genetic studies. Our investigation is one of a growing number of such studies[32,34,60–62] in which a quantitative trait or endophenotype has been helpful in understanding the role of common polymorphisms in complex behavioral disorders. A number of reviews have discussed the value of analyzing so-called QTLs in psychiatric genetics.[31,63–65] While ADHD shows substantial heritability, the molecular genetic basis remains elusive despite some recent successes, especially regarding the role of the DRD4 7 repeat.[46] One major obstacle in molecular genetic investigations has been the difficulty in

detecting nonclinically-penetrant carriers of the predisposing genes and by ambiguities concerning the nature of the non-genetic influences and the extent of locus heterogeneity. A QTL approach is an alternative method for measuring phenotypic variation that may facilitate the identification of susceptibility genes in the context of complexly-inherited traits. The current study provides additional 'proof of principle' of the QTL strategy. By evaluating the role of the MAO A long allele on a continuous performance test we extended our understanding of this gene's role in conferring risk for ADHD. Analysis of the effects of MAO A on the TOVA links this polymorphism to a behavioral phenotype, impulsivity—a core idea in ADHD. Indeed the genetic risk conferred by the MAO A long allele may be mediated by 'impulsivity.' Future studies in psychiatric genetics will undoubtedly benefit by employing both categorical and dimensional approaches to genetic studies of mental illness.

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