Association of MAOA, 5-HTT, and NET promoter polymorphisms with gene expression and protein activity in human placentas

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Zhang H, Smith GN, Liu X, Holden JJ A. Association of MAOA, 5-HTT, and NET promoter polymorphisms with gene expression and protein activity in human placentas. Physiol Genomics 42: 85–92, 2010. First published March 23, 2010; doi:10.1152/physiolgenomics.00220.2009.—Monoamine oxidase A (MAOA) and the transporters for serotonin (5-HTT) and norepinephrine (NET) may play important roles in regulating maternal monoamine neurotransmitters transferred across the placenta to the fetus. We investigated whether promoter polymorphisms in MAOA (uVNTR), 5-HTT (5-HTTLPR), and NET (NetPTP AAGG4) influence gene expression and protein activity in human placentas. Normal term human placentas (n = 73) were collected, and placental MAOA, 5-HTT, and NET mRNA levels and protein activity were determined. The mRNA levels or protein activities were compared between different genotype groups. Placentas hemizygous (male fetus) or homozygous (female fetus) for MAOA uVNTR 4-repeat allele had significantly higher MAOA mRNA levels than those hemizygous or homozygous for the 3-repeat allele (P = 0.001). However, no significant difference in MAOA enzyme activity was found for these two groups of genotypes (P = 0.161). Placentas with the 5-HTTLPR short (S)-allele (S/S + S/L) had significantly lower 5-HTT mRNA levels and serotonin uptake rate than those homozygous for the long (L)-allele (L/L) (mRNA: P < 0.001; serotonin transporting activity: P < 0.001). Placentas homozygous for the NET AAGG4 L4 allele had significantly higher NET mRNA levels, as well as dopamine and norepinephrine uptake rates, than those with the S4/L4 genotype (mRNA: P < 0.001; dopamine transporting activity: P = 0.012; norepinephrine transporting activity: P = 0.011). These findings suggest that the three promoter polymorphisms of MAOA, 5-HTT, and NET influence gene expression levels and protein activity of these genes in human placentas, potentially leading to different fetal levels of maternal monoamine neurotransmitters, which may have an impact on fetal neurodevelopment.

Monoamine oxidase A; serotonin transporter; norepinephrine transporter; neurotransmitters; mRNA levels; transport activity; fetal neuro-development

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The human NET gene (SLC6A2) is located on chromosome 16q13–21 (17). Six AAGG-repeat islands (AAGG1–6) were identified in NET promoter polymorphic region (NETpPR) (43). Among them, island AAGG4 is polymorphic. Querying the NETpPR DNA sequence against the transcription factor database TRANSFAC by the MatInspector program (33) indicated that transcription factor Elk-1 potentially binds to the region where island AAGG4 is located. The 4-bp AAGG deletion may result in the net loss of the binding site for Elk-1. However, the effect of the 4-bp AAGG deletion/insertion polymorphism in island AAGG4 on NET expression is as yet unknown.

There is evidence that the promoter polymorphisms MAOA uVNTR, 5-HTT 5-HTTLPR, and NET AAGG4 are associated with vulnerability to a number of neuropsychiatric disorders. MAOA uVNTR has been associated with impulsive/antisocial behavior (8, 12, 30, 50), autism (9, 53), affective disorders (1, 54), substance dependence (12), and posttraumatic stress disorder (51). 5-HTT 5-HTTLPR may influence anxiety-related personality traits (28, 39), affective disorders (10, 20, 25, 45), Alzheimer’s disease (34, 42), autism (5, 11, 24), sudden infant death syndrome (47), alcohol dependence (15, 31), obsessive-compulsive disorder (21), attention-deficit/hyperactivity disorder (36), and type 2 diabetes (22). NET AAGG4 has not been well studied, though a positive association between NET AAGG4 and anorexia nervosa has been reported (43). Additionally, an interactive effect of NET AAGG4 and MAOA uVNTR on anorexia nervosa was observed (44).

Since MAOA, 5-HTT, and NET are present in the placenta, altered expression and activity of these proteins due to promoter polymorphisms (MAOA uVNTR, 5-HTT 5-HTTLPR, and NET AAGG4) in their genes may influence fetal brain development. Animal studies have demonstrated that placental 5-HTT is involved in the transfer of maternal 5-HT to the fetus during the early stages of fetal development (52). The placental transporter may also participate in the localized clearance of vasoconstrictive biogenic amines such as 5-HT from the intervascular space. Insufficient clearance may lead to impaired placental blood flow and intrauterine growth retardation (16). Furthermore, studies have shown that placental transporters regulate catecholamine levels in fetal circulation by reuptake of catecholamines from the fetal plasma compartment (6). Therefore, it is important to determine whether these promoter-associated polymorphisms affect gene transcription and protein activity of the respective genes and gene products in human placentas.

**MATERIALS AND METHODS**

**Placental tissues.** Normal term (37–42 wk gestation) human placentas (n = 73) were obtained from uncomplicated pregnancies with normal outcomes from a primarily Caucasian population at the Kingston General Hospital (Ontario, Canada). None of the pregnancies were complicated (i.e., pre-eclampsia, preterm birth, intrauterine growth restriction, choorioamnionitis, stillbirth/early neonatal death). Placental tissue was processed immediately following delivery. After removal of the basal plate (including maternal decidua), the placental villous tissue (central portion of the placenta) was dissected from the chorionic plate for brush-border membrane vesicle (n = 21) and mitochondrial (n = 73) preparations and for DNA (n = 73) and total RNA (n = 73) extractions. The study protocol was approved by the institutional review boards of Queen’s University and the Kingston General Hospital.

DNA extraction and genotyping. Genomic DNA was extracted from placental tissue using QIAamp Tissue Kits (Qiagen, Valencia, CA). The DNA fragment containing promoter polymorphism MAOA uVNTR was amplified by polymerase chain reaction (PCR) as described by Sabol et al. (38) using the following primers: forward 5’-ACAGCCTGACCGTGGGAAGA-3’ and reverse 5’-GAACGGACGGCTCATTCCGA-3’. The number of 30-bp repeats was deduced from the allele size. Fetal sex was examined by amplifying two loci (ZFX and ZFY) that resulted in X-specific and Y-specific products of 488 and 340 bp, respectively (26). Based on sex information, MAOA genotypes of hemizygous male and homozygous female fetuses were distinguished.

Genotyping of 5-HTTLPR 44-bp deletion/insertion polymorphism was performed as previously described (13). 5-HTTLPR alleles were denoted as S or L. To genotype NET AAGG4, the following primers were designed: forward, 5’-GATGGAAGGAAAGGTGGAA-3’ and reverse, 5’-TCTCTTCTTCTTCTTCTC-3’ (position −4024 to −4003). Labeling PCR reactions were carried out in 10-μl volumes containing 25 ng genomic DNA, 200 μM dNTPs, 10 pmol of each primer, 0.5 μl Taq DNA polymerase (Invitrogen, Carlsbad, CA), 1× PCR reaction buffer, 1.0 mM MgCl2, and 0.1 pmol α-32P-dCTP. The PCR program was set as: 95°C for 5 min; 30 cycles of 95°C for 1 min, 67°C for 1 min, 72°C for 1 min; and finally 72°C for 10 min. PCR products were separated by electrophoresis on a 6% denaturing acrylamide gel. The 4-bp AAGG deletion allele (or L4) was 219 bp long and the 4-bp AAGG insertion allele (or S4) was 223 bp long.

**Determination of mRNA levels by quantitative duplex PCR.** Total RNA was isolated from placental tissue using TRIzol Reagent (Invitrogen). The first-strand cDNA was synthesized from 1 μg of total RNA in a 20-μl reaction with the Reverse Transcription System (Promega, Madison, WI). Relative transcription levels of MAOA, 5-HTT, and NET were determined by quantitative duplex PCRs using the β-actin gene (ACTB) as an internal control. To show unambiguously that the PCR products were derived from cDNA and not from genomic DNA, the forward and reverse primers were located in different exons, separated by at least one intron. Sequences of primers and sizes of PCR products from both genomic DNA and cDNA are given in Table 1.

Duplex-PCR amplification of MAOA and ACTB cDNAs was carried out in 10 μl of reaction mix containing 2% of the cDNA synthesized from 1 μg total RNA, 1× PCR reaction buffer, 1 mM MgCl2, 200 μM dNTPs, 0.5 μl Taq DNA polymerase (Invitrogen), 0.5 pmol MAOA forward primer, 0.49 pmol MAOA reverse primer, 0.01 pmol γ-32P end-labeled MAOA reverse primer, 0.5 pmol ACTB forward primer, 0.49 pmol ACTB reverse primer, and 0.01 pmol γ-32P end-labeled ACTB reverse primer. PCR was performed as follows: denaturation at 95°C for 5 min, followed by 20 cycles (optimized) of 95°C for 1 min, 65°C for 1 min, and 72°C for 1 min. Final extension was at 72°C for 10 min.

Duplex-PCR amplification of 5-HTT and ACTB cDNAs was carried out in 10 μl of reaction mix, containing 2% of the cDNA synthesized from 1 μg total RNA, 1× PCR reaction buffer, 1 mM MgCl2, 200 μM dNTPs, 0.5 U Taq DNA polymerase (Invitrogen), 1 pmol 5-HTT forward primer, 0.98 pmol 5-HTT reverse primer, 0.02 pmol γ-32P end-labeled 5-HTT reverse primer, 0.5 pmol ACTB forward primer, 0.49 pmol ACTB reverse primer, and 0.01 pmol γ-32P end-labeled ACTB reverse primer. Successful amplification was achieved using an initial denaturation step at 95°C for 5 min, followed by 20 cycles (optimized) of 95°C for 1 min, 65°C for 1 min, and 72°C for 1 min. Final extension was at 72°C for 10 min. duplex-PCR amplification of 5-HTT and ACTB cDNAs was performed using the same conditions as for 5-HTT cDNA, except 0.5 pmol NET forward primer, 0.45 pmol NET reverse primer, 0.05 pmol γ-32P end-labeled NET reverse primer were used in the reaction.

PCR products were separated on 6% denaturing acrylamide gels. Gels were dried and exposed to a Storage Phosphor Screen and
and filters, uptake was also measured in the presence of 100 nM desipramine, a NET inhibitor (35).

Statistical analyses. The relationship between genotypes and transcription levels or protein activities was analyzed by unpaired t-test (two-tailed) program incorporated in software GraphPad Prism 5.02 (GraphPad Software, La Jolla, CA). The comparisons were between two groups of MAOA uVNTR genotypes [3-repeat (males) + 3-repeat/3-repeat (females) vs. 4-repeat (males) + 4-repeat/4-repeat (females)], two groups of 5-HTT 5-HTTLPR genotypes (L/L vs. S/L+S/S), and two groups of NET AAGG4 genotypes (L4/L4 vs. S4/L4).

RESULTS

MAOA uVNTR genotypes and MAOA transcription level/enzyme activity. Based on the genotype of ZFX and ZFY, 34 fetuses were identified to be males and 39 fetuses were identified to be females. The 3.5-repeat allele appeared in only three placentas (two with the genotype 3-repeat/3.5-repeat and one with genotype 3.5-repeat/4-repeat) and the 5-repeat allele was found in a single placenta (genotype 3-repeat/5-repeat). The remaining 69 placentas had genotypes consisting of the two common alleles (3-repeat and/or 4-repeat). MAOA transcription levels and MAOA enzyme activity were compared between the two groups of placentas [hemizygous (male) or homozygous (female) for the 4-repeat allele]. A significant difference in relative MAOA mRNA levels (MAOA/ACTB) was found between these two groups of placentas (t = 3.44, df = 48, P = 0.001). The 4-repeat allele was associated with significantly higher mRNA levels (mean ratio ± SE: 1.14 ± 0.05) than the 3-repeat allele (mean ratio ± SE: 0.84 ± 0.06) (Table 2 and Fig. 1A). However, MAOA enzyme activity in the two groups of placentas was not significantly different (t = 1.42, df = 48, P = 0.161) (Table 2 and Fig. 1B).

5-HTTLPR genotypes and 5-HTT transcription level/transport activity. Among 73 placental DNAs, 25 had the L/L genotype, 28 had the S/L genotype, and 20 had the S/S genotype. Relative transcription levels (5-HTT/ACTB, mean ratio ± SE) associated with the L/L, S/L, and S/S genotypes were 0.65 ± 0.03 (n = 25), 0.31 ± 0.02 (n = 28), and 0.28 ± 0.01 (n = 20), respectively. Placentas with the 44-bp deletion allele (S/S and S/L) had significantly lower 5-HTT mRNA levels than those homozygous for the 44-bp insertion allele (L/L) (t = 14.29, df = 71, P < 0.001). No significant difference in 5-HTT mRNA levels was found between placentas with S/S and S/L genotypes (t = 1.25, df = 46, P = 0.218) (Table 2 and Fig. 2A).

Table 1. Location and sequence of primers for quantitative duplex PCRs and PCR product size

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Location and Sequence</th>
<th>PCR Product Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>From Genomic DNA</td>
<td>From cDNA</td>
</tr>
<tr>
<td>ACTB</td>
<td>F: 5'-AGG ACA GAG CCT GCC GCT T-3' (exon 1)</td>
<td>1,343</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CAG AGG CAG CTC ATT GTA GA-3' (exon 3)</td>
<td>2,6370</td>
</tr>
<tr>
<td>MAOA</td>
<td>F: 5'-TGTC TGC CAA ACT CCT GAC TG-3' (exon 2)</td>
<td>2,519</td>
</tr>
<tr>
<td>5-HTT</td>
<td>F: 5'-AGG TGG AAC GAC TCC TGG AA-3' (exon 3)</td>
<td>2,467</td>
</tr>
<tr>
<td></td>
<td>R: 5'-ATG TGA GGC GGT GCA GCT AT-3' (exon 6)</td>
<td></td>
</tr>
<tr>
<td>NET</td>
<td>F: 5'-TTT GGA AAA TCT GCC CAT TC-3' (exon 2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: 5'-TAC TTG GTG TGG TGG CCA AG-3' (exon 3)</td>
<td></td>
</tr>
</tbody>
</table>

quantified on a Molecular Dynamics Phosphor Imager (Amersham Pharmacia Biotech, Piscataway, NJ). Counts per minute of each band in uniformly sized sections was measured. Incorporation ratios (MAOA/ACTB, 5-HTT/ACTB, and NET/ACTB) for each cDNA sample were calculated and viewed as relative transcription levels for each of the three tested genes. The assays were done in duplicate for each sample.

Mitochondria isolation and assays of MAOA enzyme activity. Isolation of mitochondria from placental tissue and the assay of MAOA activity were based on the method described by Weyler and Salach (49). The measurement of MAOA enzyme activity was carried out in 50 mM sodium phosphate buffer, pH 7.2, containing 0.2% Triton X-100 (monoamine oxidase assay buffer), plus 2 mM of kynuramine (substrate for MAOA), and an appropriate amount of the mitochondrial supernatant (optimized to be 100 ng of protein in the supernatant). The mixture was incubated at room temperature for 30 min, and the OD value at 314 nm (specific absorbency peak for the product, 4-hydroxyquinoline) was recorded. One unit of active enzyme equals to the amount of enzyme protein forming 1 μmol of product/min under these assay conditions. Enzyme assay was performed in duplicate for each sample.

Brush-border membrane vesicle preparation and 5-HTT and NET activity assay. Brush-border membrane vesicles from 21 placentas were prepared according to the method described previously (4). The activity of 5-HTT and NET in brush border membrane vesicles was determined by measuring the uptake of H+ labeled monoamines (5-HT, DA, or NE) in the presence of an inwardly directed sodium chloride gradient. Uptake measurements were made by a rapid filtration technique as previously described (35). Briefly, uptake was initiated by rapidly mixing 40 μl of the membrane suspension (0.24 mg of protein) with 160 μl of uptake buffer (20 mM MES/Tris, 140 mM NaCl, pH 6.5) containing H+ labeled monoamines. After 15 s incubation at room temperature, uptake was terminated by adding 3 ml of ice-cold stop buffer (5 mM MES/Tris, 160 mM KCl, pH 6.5), and the mixture was filtered. Membrane vesicles retained in the filter were washed three times with 3 ml of the stop buffer. The filter was then placed in a 5-ml counting vial containing 2.5 ml of liquid scintillation cocktail CytoScount ES (ICN Pharmaceuticals, Costa Mesa, CA). The radioactivity associated with the filter was measured using a Beckman Model LS-230 liquid scintillation spectrometer (Beckman Coulter, Brea, CA). The experiments were performed in triplicate.

To determine the uptake rate by 5-HTT, 5-[1,2-3H(N)]-hydroxytryptamine creatinine (H+5-HT) (PerkinElmer, Waltham, MA) was used as substrate (final concentration in the mixture: 25 nM). To adjust for nonspecific binding of the radio-label to membrane vesicles and filters, uptake was also measured in the presence of 100 μM imipramine, a 5-HTT inhibitor (4). Two other substrates, 3,4-[7-3H]-dihydroxyphenylethylamine (H+DA) and levo-[7-3H]-norepinephrine (H+NE) (PerkinElmer), were used to determine NET transport activity. Their final concentrations were 50 and 200 nM, respectively. To adjust for nonspecific binding of the radio-label to membrane vesicles and the filter, uptake was also measured in the presence of 100 μM desipramine, a NET inhibitor (35).
5-HTT activity in 21 placentas was determined by uptake measurements using H$^3$-5-HT as the substrate. The mean (± SE) uptake rates associated with the three genotypic groups were 1.85 ± 0.01 (L/L, n = 4), 1.55 ± 0.02 (S/L, n = 9), and 1.53 ± 0.02 (S/S, n = 8) pmol/mg protein/15 s, respectively. Placentas with the L/L genotype had a significantly higher uptake rate than those with the 44-bp deletion allele (S/L and S/S genotypes) (t = 9.66, df = 19, P < 0.001). However, there was no significant difference in the uptake rate between placentas with S/S and S/L genotypes (t = 0.77, df = 15, P = 0.450) (Table 2 and Fig. 2B).

**NET AAGG₄ genotypes and NET transcription level/transport activity.** Genotyping the NET AAGG₄ polymorphism indicated that 47 placentas were homozygous for the 4-bp AAGG insertion allele (L₄/L₄), 25 were heterozygous (S₄/L₄), and 1 was homozygous for the 4-bp AAGG deletion allele (S₄/S₄). Relative NET mRNA levels (NET/ACTB) (mean ratio ± SE) corresponding to the three groups of genotypes were 0.53 ± 0.02 (47 L₄/L₄), 0.22 ± 0.02 (25 S₄/L₄), and 0.09 (1 S₄/S₄), respectively. NET mRNA levels were significantly higher in placentas with the L₄/L₄ genotype than in those with the S₄/L₄ genotype (t = 8.46, df = 70, P < 0.001) (Table 2 and Fig. 3A). Since only one placenta was homozygous for the deletion allele (S₄/S₄), genotype S₄/S₄ was not included in the analysis.

Transport activity of NET in 21 placentas was determined by uptake of H$^3$DA as a substrate. The mean (± SE) transport activities (pmol/mg protein/15 s) were 2.34 ± 0.03 and 2.18 ± 0.05 for the uptake of H$^3$DA, and 4.29 ± 0.06 and 4.00 ± 0.10 for the uptake of H$^3$-NE, respectively, for the uptake of H$^3$-DA, and 4.29 ± 0.06 and 4.00 ± 0.10 for the uptake of H$^3$-NE, respectively, for the uptake of H$^3$-NE. Transport was more efficient by placentas with the L₄/L₄ genotype compared with those with the S₄/L₄ genotype (Table 2).

**Table 2. Association of MAOA, 5-HTT, and NET genotypes with transcription levels and protein activities in human placentas**

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Relative mRNA Levels, mean ± SE</th>
<th>Protein Activities, mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAOA uVNTR</td>
<td>MAOA/ACTB</td>
<td>Enzyme activity, units/g protein</td>
</tr>
<tr>
<td>3-repeat</td>
<td>0.85 ± 0.07 (n = 12 males)</td>
<td>11.20 ± 0.28 (n = 12 males)</td>
</tr>
<tr>
<td>3-repeat/3-repeat</td>
<td>0.80 ± 0.12 (n = 5 females)</td>
<td>11.17 ± 0.41 (n = 5 females)</td>
</tr>
<tr>
<td>4-repeat</td>
<td>1.21 ± 0.05 (n = 22 males)</td>
<td>11.78 ± 0.20 (n = 22 males)</td>
</tr>
<tr>
<td>4-repeat/4-repeat</td>
<td>1.01 ± 0.12 (n = 11 females)</td>
<td>11.12 ± 0.11 (n = 11 females)</td>
</tr>
<tr>
<td>3-repeat +3-repeat/3-repeat</td>
<td>0.84 ± 0.06 (n = 17)</td>
<td>11.19 ± 0.22 (n = 17)</td>
</tr>
<tr>
<td>vs. 4-repeat +4-repeat/4-repeat</td>
<td>1.14 ± 0.05 (n = 33)</td>
<td>11.56 ± 0.14 (n = 33)</td>
</tr>
<tr>
<td></td>
<td>t = 3.45, df = 48, P = 0.001</td>
<td>t = 1.42, df = 48, P = 0.161</td>
</tr>
<tr>
<td>5-HTT 5-HTTLPR</td>
<td>5-HT /ACTB</td>
<td>5-HT uptake rate, pmol/mg protein/15 s</td>
</tr>
<tr>
<td>S/S</td>
<td>0.28 ± 0.01 (n = 20)</td>
<td>1.53 ± 0.02 (n = 8)</td>
</tr>
<tr>
<td>vs. S/I</td>
<td>0.31 ± 0.02 (n = 28)</td>
<td>1.55 ± 0.02 (n = 9)</td>
</tr>
<tr>
<td>L/I</td>
<td>0.65 ± 0.03 (n = 25)</td>
<td>1.85 ± 0.01 (n = 4)</td>
</tr>
<tr>
<td>vs. S/S + S/I</td>
<td>0.29 ± 0.01 (n = 48)</td>
<td>1.54 ± 0.01 (n = 17)</td>
</tr>
<tr>
<td>t = 14.29, df = 71, P &lt; 0.001</td>
<td>t = 9.66, df = 19, P &lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>NET AAGG₄</td>
<td>NET/ACTB</td>
<td>DA or NE uptake rate, pmol/mg protein/15 s</td>
</tr>
<tr>
<td>S₄/S₄</td>
<td>0.09 (n = 1)</td>
<td>N/A</td>
</tr>
<tr>
<td>vs. S₄/I</td>
<td>0.22 ± 0.02 (n = 25)</td>
<td>2.18 ± 0.05 (n = 7, DA uptake)</td>
</tr>
<tr>
<td>vs. L₄/I</td>
<td>0.53 ± 0.02 (n = 47)</td>
<td>4.00 ± 0.10 (n = 7, NE uptake)</td>
</tr>
<tr>
<td>t = 8.46, df = 70, P &lt; 0.001</td>
<td>t = 2.77, df = 19, P = 0.012 (DA)</td>
<td></td>
</tr>
<tr>
<td>S₄/I vs. L₄/I</td>
<td>t = 2.80, df = 19, P = 0.011 (NE)</td>
<td></td>
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</table>

**DISCUSSION**

Expression of MAOA, 5-HTT, and NET in placental tissue suggests that these proteins may be involved in the regulation of monoamine neurotransmitter levels in the fetal circulation. Altered activities of these proteins in placentas may thus have a direct influence on fetal growth and development. For example, reduced placental MAOA activity and elevated maternal 5-HT levels have been implicated in pre-eclampsia (7). In the present study, we examined the effect of three promoter-associated polymorphisms (MAOA uVNTR, 5-HTT 5-HTTLPR, and NET AAGG₄) on gene expression and protein activity in human placentas.

Our study demonstrates that the MAOA uVNTR promoter polymorphism affects MAOA transcription in human placentas. Alleles with four copies of the 30-bp repeat were transcribed more efficiently than alleles with three copies of the repeat. This result is consistent with that obtained from in vitro transfection experiments (14, 38), and indicates that 4-repeat alleles have an optimal length for maintaining MAOA promoter activity. Despite the transcription difference, placentas with the 4-repeat allele did not show significantly higher MAOA enzyme activity than those with the 3-repeat allele. First, this may be due to the limited sample size, which did not have enough power to detect a small but significant difference between the two groups of genotypes. Second, proteins go through a series of post-translational modifications (e.g., NH₂-terminal signal peptide cleavage, disulfide bond generation, glycosylation, and sterol structure formation, etc) before they attain biological function. There may be limits to any of these processes that

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Affect the amount of functional protein even if the amount of translated products differed in placentas with different genotypes. Third, endogenous enzyme inhibitors may affect MAOA enzyme activity. Sivasubramaniam et al. (40) examined MAOA expression and activity in human placentas from pre-eclamptic and normotensive pregnancies and found that the catalytic efficiency of MAOA enzyme in pre-eclamptic placentas is significantly reduced, but MAOA mRNA levels and MAOA protein/mg total protein are not changed. Decreased MAOA enzyme activity may result from the presence of endogenous enzyme inhibitors that are activated in conditions such as pre-eclampsia. Fourth, placental MAOA expression and activity may vary based on the stage of fetal development. Developmental expression of MAOA has been observed in mouse brain. Strong MAOA expression can be detected in serotoninergic neurons of the raphe nuclei from E12 to P7. During postnatal life, MAOA expression declines progressively to reach a minimal stable level by P21 (46). This may happen in the placenta as well. During later stages of pregnancy, the fetus may start to produce its own monoamine neurotransmitters. The maternal monoamine neurotransmitters would then no longer be as important as in the early stages of pregnancy. Correspondingly, the regulatory effect of gene variation on placental MAOA expression and activity may become unnoticeable. Finally, in addition to MAOA, other enzymes (e.g., catechol-O-methyltransferase or COMT) metabolize monoamines. A study by Wang et al. (46a) showed that COMT was more important than MAO in metabolizing intrarenal dopamine, suggesting that the enzyme activity of MAOA may be compensated for by other enzymes. Therefore, future studies should examine whether the interaction of monoamine metabolizing enzymes in the placenta regulates the transfer of maternal monoamines to the fetus.

With regard to the promoter polymorphism 5-HTTLPR, the 44-bp deletion allele (or S-allele) was associated with a decreased transcription level of 5-HTT and a low level of 5-HTT.

**Fig. 1.** Association of MAOA uVNTR genotypes and MAOA mRNA level/enzyme activity. ■ Relative MAOA mRNA level/enzyme activity in placentas with only the 3-repeat allele (n = 17), ▲ Relative MAOA mRNA level/enzyme activity in placentas with only with the 4-repeat allele (n = 33). Horizontal lines represent mean values.

**Fig. 2.** Association of 5-HTTLPR genotypes and 5-HTT mRNA level/transport activity. Each square indicates relative 5-HTT mRNA level/transport activity in placentas with the L/L genotype (n = 25 for mRNA, n = 4 for protein). ▲ Relative 5-HTT mRNA level/transport activity in placentas with the S/L genotype (n = 28 for mRNA, n = 9 for protein). ▼ Relative 5-HTT mRNA level/transport activity in placentas with the S/S genotype (n = 20 for mRNA, n = 8 for protein). 5-HTT transport activity was measured in 21 placentas by uptake experiments with substrate H3-5-HT. The horizontal lines represent mean values.
transport activity in human placentas. The dominant effect of the S-allele on 5-HTT expression was also found for human platelets and lymphoblasts (2, 18, 28). However, it is unknown why the influence of the L-allele on 5-HTT promoter activity is suppressed in heterozygous (S/L) subjects. A possible reason is that the function of the L allele is silenced due to genomic imprinting, in which DNA methylation may play a key role (37). Further studies are warranted.

The present study provides the first evidence that the NET AAGG4 promoter polymorphism is a functional variant or in close linkage disequilibrium with a functional variant. The AAGG4 4-bp deletion allele (S4) is associated with low transcription levels and low transport activity for both DA and NE. However, it is uncertain whether the S4-allele exerts a dominant effect on NET expression because only one placenta was homozygous for the deletion allele (S4/S4). Nevertheless, the transcription level in that placental tissue (0.09) was much lower than that associated with heterozygous genotype S4/L4 (0.22 ± 0.09). This may be because the deletion of the 4-bp AAGG on both alleles leads to the loss of a binding site for a putative transcription factor Elk-1, thus substantially decreasing NET transcription. Stober et al. (41) investigated a highly polymorphic silent polymorphism 1287G/A in NET to distinguish the expression of maternal and paternal alleles in adult human brain. Their results suggest that NET is not imprinted in brain tissues and is thus biallelic in terms of expression. If biallelic expression of NET is indeed shown in placental tissue, then it can be deduced that genotypes S4/S4, S4/L4, and L4/L4 are associated with the lowest, moderate, and highest transcription levels, respectively.

There are several strengths to our experimental design. First, the present study is the first to address the relationship between MAOA, 5-HTT, and NET genotypes and transcription levels and protein activities in human placentas obtained following uncomplicated term pregnancies. Second, the quantitative duplex PCR method applied in this study is more accurate than that used by Sivasubramaniam et al. (40). In their study, amplification of MAOA and the internal control, ACTB, were performed in separate reactions using different amounts of template cDNA and different numbers of PCR cycles. Small variations in cDNA template may lead to biased transcription levels of the target gene. Lastly, our study, for the first time, demonstrated that the NET AAGG4 promoter polymorphism is likely functional and that the 4-bp AAGG deletion may result in decreased NET promoter activity.

This study demonstrated that transcription levels of MAOA, and both transcription and protein activities of 5-HTT and NET, in the placenta are regulated by sequence variation in the promoter region of the three genes. In future studies, the effects of other polymorphisms in MAOA, 5-HTT, and NET should be studied as they may be in close linkage disequilibrium with the above promoter polymorphisms or they may be the true variants that affect gene transcription and protein activity.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.


