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Enhancing genotyping of *MAOA*-LPR and *5-HTT*-LPR in rhesus macaques (*Macaca mulatta*)

Genesio M. Karere^{1,3,*}, Erin Sullivan^{2,3}, Erin L. Kinnally³, John P. Capitanio^{2,3}, and Leslie A. Lyons^{1,3}

¹Department of Population Health and Reproduction, University of California-Davis, Davis, CA 95616

²Department of Psychology, University of California-Davis, Davis, CA 95616

³California National Primate Research Center, University of California-Davis, Davis, CA 95616

Abstract

Background—Genetic variation in *monoamine oxidase A* (*MAOA*) and *serotonin transporter* (*5-HTT*) linked polymorphic regions (LPR) is associated with neuropsychiatric behavior.

Method—We genotyped 37 macaques using conventional PCR-product gel fractionation and by capillary electrophoresis of multiplexed amplicons and compared the data.

Results—Genotype concordance was 97% and 95% for *MAOA*-LPR and *5-HTT*-LPR, respectively. Capillary electrophoresis was more sensitive and cost-effective.

Conclusions—Multiplexing *MAOA*-LPR and *5-HTT*-LPR will enhance the genotyping of large sample sets.

Keywords

monoamine oxidase A; *serotonin transporter*; multiplexing; capillary electrophoresis; rhesus macaque

Introduction

The serotonin system is an important neurophysiological mediator of many behavioral phenotypes. Serotonin (5-hydroxytryptamine) transporter (*5-HTT*) and monoamine oxidase A (*MAOA*) encode two of the major regulatory proteins in the serotonergic pathway. *MAOA* is X-linked and encodes an enzyme that catalyzes the oxidation of serotonin (5-HT), dopamine (DA), and norepinephrine (NE) neurotransmitters. *5-HTT* gene (SLC6A4) is localized on human chromosome 17 and transcribes a protein involved in the re-uptake of 5-HT from the synaptic cleft, into the pre-synaptic neuron. Functional polymorphisms in the promoter linked polymorphic region (LPR) of *MAOA* and *5-HTT* have been widely studied in humans and non-human primates [2, 6, 15]. These polymorphisms include 18 bp untranslated variable number tandem repeats (uVNTR) for rhesus macaque (rh) *MAOA* and an insertion or deletion (indel) of a 21 bp fragment for rh *5-HTT*. The uVNTR includes 5, 6, or 7-repeats that confer differential transcriptional efficiencies (5 and 6 repeats have high transcriptional activity, 7 repeat has low activity) of the *MAOA* gene [15]. For *5-HTT*-LPR, individuals with the insertion fragment are considered to have the long allele (high activity),

*Corresponding author. Current address: Texas Biomedical Research Institute, Department of Genetics, 7620 NW Loop 410, San Antonio, TX 78227-5301, Tel. (210) 258-9622, Fax (210) 258-9131, gmkarere@txbiomedgenetics.org.

whereas those without the insertion are discerned as possessing the short allele (low activity) [10].

Several studies have demonstrated that these polymorphisms are associated with psychological dysfunction including depression [16], anxiety [9], autism [17], schizophrenia [20], addiction disorders [14] eating disorders [13], compulsive shopping [17], impulsivity [19], and numerous substance abuse disorders [11]. In addition, the effects of gene-gene and gene-environment interactions on later behavior have been investigated in primates [4–8]. However, results of some primate studies do not show consistency, possibly reflecting sample size differences, environmental factors, or, other genetic influences.

One of the challenges of investigating the effects of both *MAOA-LPR* and *5-HTT-LPR* on behavioral variation is the ability to genotype a large number of samples efficiently to increase the statistical power of prediction. Most studies have used a conventional PCR-based method to amplify the marker-specific polymorphic regions, followed by enzyme restriction of the PCR products and/or separation by electrophoresis on gel matrices. Because of low resolution and sensitivity, the conventional PCR-based method is susceptible to technical errors and experimenter variability. Due to these reasons, duplicate or triplicate typing is practiced. Accordingly, the conventional method is slow, expensive, and limits data generation. A few studies have used labeled primers to genotype *5-HTT-LPR* [18]; however, to our knowledge, no study reports a high-throughput method for multiplexing *MAOA-LPR* and *5-HTT-LPR*, major genetic polymorphism commonly analyzed in many behavioral studies.

Materials and Methods

Sample collection and DNA extraction

The University of California-Davis, Institutional Animal Care and Use Committee approved sample collection procedures. Blood samples were collected and prepared as described [6]. In brief, staff veterinarians of the California National Primate Research Center collected blood samples (1ml), through the femoral vein, from ketamine-sedated three to four months-old rhesus macaque (*Macaca mulatta*) infants. In this study, 37 samples (females, n = 18, males, n = 19) were processed by centrifugation at 12,000 g for 10 min and buffy coats aspirated for DNA extractions. DNA was extracted using a standard phenol-chloroform protocol and quantified using NanoDrop 1000 spectrophotometer (Thermal Scientific, Wilmington, DE). Briefly, the buffy coat was washed with 1X PBS and red blood cells lysed with 1X Lysis buffer at room temperature for 15 min. The mixture was centrifuged at 12,000 g for 5 min and supernant discarded. To lyse the nucleated cells, 1X Lysis buffer containing 2% SDS and Proteinase K was added to the pellet and incubated at 37°C overnight. For DNA extraction, phenol:chloroform (1:1) was added to the mixture, vortexed and centrifuged at 12,000 g for 10 min. The upper aqueous layer was carefully aspirated and transferred to a new tube. The phenol:chloroform extraction step was repeated and DNA precipitated by adding 3M sodium acetate and 95% ice-cold ethanol, mixed and centrifuged at 12,000 g for 1 min. DNA pellet was washed with 75% ethanol, spun down and air-dried at room temperature. The dried pellet was suspended in 1X TE buffer prior to quantification.

Genotyping

Genotyping for rh *MAOA-LPR* and *5-HTT-LPR* was performed by fractionating PCR products in 2% agarose gels and by capillary electrophoresis in ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA). Primer pairs for *MAOA* (Forward: CAGAAACATGAGCACAAACG, Reverse: TACGAGGTGTCGTCCAAGTT) and *5-HTT*

(Forward: GCCGCTCTGAATACCAGCAC, Reverse: GGAGGGATGCAGGGGTTG) were designed from the promoter sequences of the rh *MAOA* (Accession number AJ544234) and *5-HTT* (AF285761) available in GenBank database [3].

For the PCR, we used the following amplification profile: denaturation for 5 min at 95°C, followed by 40 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72 °C and a final extension for 10 min at 72°C. Each PCR reaction (15ul) contained 60 ng of DNA, 1X PCR buffer (ABgene, Rochester, NY) containing 5% BSA, 0.2 mM dNTP, 1.5 mM Betaine, 1.5 mM MgCl₂, and 0.25 U Taq polymerase (Denville Scientific Inc., Metuchen, NJ). Primers in the reaction included a mixture of 0.7 uM *MAOA*-specific primer mix and 0.1 uM *5-HTT*-specific primer mix. Forward primer for *MAOA-LPR* was fluorescently labeled at the 5' end with FAM dye, and *5-HTT-LPR* reverse primer with PET dye (Applied Biosystems, Foster City, CA).

For amplicon size-separation by capillary electrophoresis, 3ul of PCR-product dilute (1:200) was mixed with 9ul of LIZ-500™ size-standard and Hi-Di™ Formamide master mix (Applied Biosystems, Foster City, CA). The mixture was briefly vortexed and spun down. Finally the sample was denatured at 95°C for 4 min and chilled on ice for 2 min prior to loading onto AB3730 DNA Analyzer. Genotypes were analyzed using STRand [21]. Due to the size-standard used in the AB3730 DNA Analyzer, the *MAOA-LPR* allele sizes shifted consistently by 6 bp from the previously established sequence sizes [12]. Thus, *MAOA-LPR* allele-calling was adjusted to conform to the sequence results by adding 6 base pairs (bp) for each of the three alleles.

For the agarose gel electrophoresis, each PCR reaction contained in addition either 1uM of primer mix for *MAOA-LPR* or *5-HTT-LPR*. Gel electrophoresis was performed at 100 volts for 1 hr, amplicons visualized under UV light and sized using a 1 Kb Plus DNA Ladder (Invitrogen, San Diego, CA).

Sequencing

Allelic sizes and identity were confirmed by direct sequencing on an ABI 3730 DNA Analyzer using BigDye Terminator Sequencing chemistry v3.1 (Applied Biosystems) as described elsewhere [12]. The forward and reverse DNA sequences were aligned using Sequencer Software (Gene Codes, Ann Arbor, MI) and confirmed to be the correct gene using BLAST [1] to the GenBank database.

Results and Discussion

We compared data generated from 37 rhesus macaque DNA samples by both the high-throughput capillary electrophoresis of multiplexed amplicons and the conventional PCR-product gel fractionation method. The allelic variants observed included 246, 264, and 282 bp for rh *MAOA-LPR*, corresponding to 5-, 6-, and 7- repeats. *5-HTT-LPR* alleles include 402 bp (long allele) and 381 bp (short allele) (Fig. 1 and 2). These results confirm previous observations [10, 15]. A remarkable genotype concordance was observed; 95% for *5-HTT-LPR* and 97% for *MAOA-LPR* (Table 1) between high-throughput and conventional method. One sample from a male subject showed genotype discordances for *MAOA-LPR* between datasets. The conventional method indicated a heterozygote genotype (264/282 bp) for this sample. Since *MAOA* is localized on X-chromosome, a heterozygous result was unexpected. In contrast the high-throughput method showed this particular sample to be homozygote (264/264 bp) as expected. The discordant result could be due to sample mix-up rather than a technical error. For *5-HTT-LPR* genotypes, we observed that two sample genotypes were discordant between the datasets; for both samples the conventional method revealed homozygous genotypes (1/1) while the high-throughput method indicated

heterozygous genotypes (l/s) (Table 1), which was confirmed by sequencing. Together our results indicate that the high-throughput method is more sensitive and accurate in genotyping.

We conclude that there are significant advantages in the use of the high-throughput genotyping method. Most importantly, because fluorescent polyacrylamide gel electrophoresis (PAGE) is automated in AB3730 DNA Analyzer and has better resolution than agarose gels used in conventional genotyping, the high-throughput method is more accurate and sensitive. Additionally, the cost and time for genotyping are significantly reduced enabling typing of thousands of samples for both *MAOA-LPR* and *5-HTT-LPR* in a short amount of time. The new protocol for genotyping *MAOA-LPR* and *5-HTT-LPR* will enhance large-scale investigations on gene-environment interactions using rhesus macaque model for human neuropsychiatric behavior.

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References

1. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J.Mol.Biol.* 1990; 215:403–410. [PubMed: 2231712]
2. Bennett AJ, Lesch KP, Heils A, Long JC, Lorenz JG, Shoaf SE, Champoux M, Suomi SJ, Linnoila MV, Higley JD. Early experience and serotonin transporter gene variation interact to influence primate CNS function. *Mol.Psychiatry.* 2002; 7:118–122. [PubMed: 11803458]
3. Benson DA, Boguski MS, Lipman DJ, Ostell J, Ouellette BF, Rapp BA, Wheeler DL. GenBank. *Nucleic Acids Res.* 1999; 27:12–17. [PubMed: 9847132]
4. Caspi A, McClay J, Moffitt TE, Mill J, Martin J, Craig IW, Taylor A, Poulton R. Role of genotype in the cycle of violence in maltreated children. *Science.* 2002; 297:851–854. [PubMed: 12161658]
5. Geller B, Cook EH Jr. Serotonin transporter gene (HTTLPR) is not in linkage disequilibrium with prepubertal and early adolescent bipolarity. *Biol.Psychiatry.* 1999; 45:1230–1233. [PubMed: 10331118]
6. Karere GM, Kinnally EL, Sanchez JN, Famula TR, Lyons LA, Capitanio JP. What is an "adverse" environment? Interactions of rearing experiences and MAOA genotype in rhesus monkeys. *Biol.Psychiatry.* 2009; 65:770–777. [PubMed: 19103441]
7. Kim-Cohen J, Caspi A, Taylor A, Williams B, Newcombe R, Craig IW, Moffitt TE. MAOA, maltreatment, and gene-environment interaction predicting children's mental health: new evidence and a meta-analysis. *Mol.Psychiatry.* 2006; 11:903–913. [PubMed: 16801953]
8. Kinnally EL, Karere GM, Lyons LA, Mendoza SP, Mason WA, Capitanio JP. Serotonin pathway gene-gene and gene-environment interactions influence behavioral stress response in infant rhesus macaques. *Dev.Psychopathol.* 2010; 22:35–44. [PubMed: 20102645]
9. Leonardo ED, Hen R. Genetics of affective and anxiety disorders. *Annu.Rev.Psychol.* 2006; 57:117–137. [PubMed: 16318591]
10. Lesch KP, Meyer J, Glatz K, Flugge G, Hinney A, Hebebrand J, Klauck SM, Poustka A, Poustka F, Bengel D, Mossner R, Riederer P, Heils A. The 5-HT transporter gene-linked polymorphic region (5-HTTLPR) in evolutionary perspective: alternative biallelic variation in rhesus monkeys. Rapid communication. *J.Neural Transm.* 1997; 104:1259–1266. [PubMed: 9503271]
11. Lin Z, Madras BK. Human genetics and pharmacology of neurotransmitter transporters. *Handb.Exp.Pharmacol.* 2006; (175):327–371. [PubMed: 16722243]
12. Lyons LA, Biller DS, Erdman CA, Lipinski MJ, Young AE, Roe BA, Qin B, Grahm RA. Feline polycystic kidney disease mutation identified in PKD1. *J.Am.Soc.Nephrol.* 2004; 15:2548–2555. [PubMed: 15466259]

13. Matsushita S, Nakamura T, Nishiguchi N, Higuchi S. Association of serotonin transporter regulatory region polymorphism and abnormal eating behaviors. *Mol.Psychiatry*. 2002; 7:538–540. [PubMed: 12140775]
14. Muller CP, Carey RJ, Huston JP, De Souza Silva MA. Serotonin and psychostimulant addiction: focus on 5-HT1A-receptors. *Prog.Neurobiol*. 2007; 81:133–178. [PubMed: 17316955]
15. Newman TK, Syagailo YV, Barr CS, Wendland JR, Champoux M, Graessle M, Suomi SJ, Higley JD, Lesch KP. Monoamine oxidase A gene promoter variation and rearing experience influences aggressive behavior in rhesus monkeys. *Biol.Psychiatry*. 2005; 57:167–172. [PubMed: 15652876]
16. Owens MJ, Nemeroff CB. Role of serotonin in the pathophysiology of depression: focus on the serotonin transporter. *Clin.Chem*. 1994; 40:288–295. [PubMed: 7508830]
17. Pardo CA, Eberhart CG. The neurobiology of autism. *Brain Pathol*. 2007; 17:434–447. [PubMed: 17919129]
18. Ramasubbu R. Antidepressant treatment-associated behavioural expression of hypomania: a case series. *Prog.Neuropsychopharmacol.Biol.Psychiatry*. 2004; 28:1201–1207. [PubMed: 15610935]
19. Sakado K, Sakado M, Muratake T, Mundt C, Someya T. A psychometrically derived impulsive trait related to a polymorphism in the serotonin transporter gene-linked polymorphic region (5-HTTLPR) in a Japanese nonclinical population: assessment by the Barratt impulsiveness scale (BIS). *Am.J.Med.Genet.B.Neuropsychiatr.Genet*. 2003; 121B:71–75. [PubMed: 12898578]
20. Semwal P, Prasad S, Varma PG, Bhagwat AM, Deshpande SN, Thelma BK. Candidate gene polymorphisms among North Indians and their association with schizophrenia in a case-control study. *J.Genet*. 2002; 81:65–71. [PubMed: 12532038]
21. Toonen RJ, Hughes S. Increased throughput for fragment analysis on an ABI PRISM 377 automated sequencer using a membrane comb and STRand software. *BioTechniques*. 2001; 31:1320–1324. [PubMed: 11768661]

Figure 1. PCR product gel electrophoresis

A) Homozygous *MAOA*-LPR alleles [M indicates 1kb+ marker, lane 1 represents a 7-repeat allele of 282 bp, 2 = 6-repeat allele (264 bp) and 3 = 5-repeat allele (246 bp)], B) Long and short alleles of serotonin transporter (*5-HTT*-LPR) [lane 1 is a sample from an heterozygous animal with short (381 bp) and long (402 bp) alleles, lane 2 = homozygous long allele, 3= heterozygous genotype, and 4 = homozygous long allele]. PCR products were separated in 2% agarose gels with ethidium bromide and visualized under UV light.

Figure 2. An electropherogram

Shows peaks of heterozygous *MAOA*-LPR (blue) and *5-HTT*-LPR (red) alleles multiplexed using AB3730 DNA Analyzer fluorescence technology and displayed using STRand software. LIZ-500™ size-standard was used.

Table 1

MAOA-LPR and 5-HTTLPR genotypes resulting from the application of the conventional PCR method and ABI3730 DNA Analyzer.

Animal Information		MAOA (No. of repeats) ¹		Serotonin transporter (long (l)/short (s) allele)	
Animal ID	Gender	Conventional PCR	ABI3730 DNA Analyzer	Conventional PCR	ABI3730 DNA Analyzer
33497	M	6	6	l/l	l/l
33096	M	7	7	l/l	l/l
33693	F	5/6	5/6	l/l	l/l
33926 ²	F	6/6	6/6	l/l	l/s
33045	F	5/6	5/6	l/l	l/l
34249	M	7	7	s/s	s/s
33648	M	5	5	l/s	l/s
33695	F	5/5	5/5	s/s	s/s
34114	M	7	7	l/l	l/l
34712	F	5/5	5/5	l/l	l/l
34671	F	7/7	7/7	l/l	l/l
34944	M	6	6	l/l	l/l
35075	M	5	5	l/l	l/l
34743	M	7	7	s/s	s/s
34763	M	5	5	l/l	l/l
32802 ²	F	5/7	5/7	l/l	l/s
32968	M	6	6	l/l	l/l
33167	F	5/7	5/7	s/s	s/s
34269	F	7/7	7/7	s/s	s/s
34651	M	6	6	l/l	l/l
35147	M	7	6	s/s	s/s
32907	M	7	7	l/l	l/l
35185 ²	M	6/7	6	l/l	l/l
33654	F	5/7	5/7	l/l	l/l
34988	F	5/7	5/7	l/l	l/l

Animal Information		MAOA (No. of repeats) ¹		Serotonin transporter (long (l)/short (s) allele)	
Animal ID	Gender	Conventional PCR	AB3730 DNA Analyzer	Conventional PCR	AB3730 DNA Analyzer
34037	M	5	5	s/s	s/s
34332	M	7	7	l/l	l/l
34739	F	5/7	5/7	s/s	s/s
34759	F	7/7	7/7	s/s	s/s
33666	M	5	5	l/l	l/l
33708	F	6/6	6/6	l/l	l/l
32938	F	5/7	5/7	l/s	l/s
33671	M	5	5	l/l	l/l
33725	M	6	6	l/l	l/l
33867	F	5/6	5/6	l/l	l/l
35005	F	6/7	6/7	l/l	l/l
35271	F	5/6	5/6	l/l	l/l

¹ Males are hemizygous for *MAOA*.

² Indicates samples discordant for *MAOA-LPR* and/ *5-HTT-LPR* genotypes.