Multiple Regulatory Variants Modulate Expression of 5-Hydroxytryptamine 2A Receptors in Human Cortex

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Background: The 5-hydroxytryptamine 2A receptor, encoded by *HTR2A*, is a major postsynaptic target for serotonin in the human brain and a therapeutic drug target. Despite hundreds of genetic associations investigating *HTR2A* polymorphisms in neuropsychiatric disorders and therapies, the role of genetic *HTR2A* variability in health and disease remains uncertain.

Methods: To discover and characterize regulatory *HTR2A* variants, we sequenced whole transcriptomes from 10 human brain regions with massively parallel RNA sequencing and measured allelic expression of multiple *HTR2A* messenger (m)RNA transcript variants. Following discovery of functional variants, we further characterized their impact on genetic expression in vitro.

Results: Three polymorphisms modulate the use of novel alternative exons and untranslated regions (UTRs), changing expression of RNA and protein. The frequent promoter variant rs6311, widely implicated in human neuropsychiatric disorders, decreases usage of an upstream transcription start site encoding a longer 5′UTR with greater translation efficiency. rs76665058, located in an extended 3′UTR and unique to individuals of African descent, modulates allelic *HTR2A* mRNA expression. The third single nucleotide polymorphism, unannotated and present in only a single subject, directs alternative splicing of exon 2. Targeted analysis of *HTR2A* in the Sequenced Treatment Alternatives to Relieve Depression (STAR*D) study reveals associations between functional variants and depression severity or citalopram response.

Conclusions: Regulatory polymorphisms modulate *HTR2A* mRNA expression in an isoform-specific manner, directing the usage of novel untranslated regions and alternative exons. These results provide a foundation for delineating the role of *HTR2A* and serotonin signaling in central nervous system disorders.

Key Words: 5-HT2A, depression, HTR2A, mRNA expression, schizophrenia, serotonin

The 5-hydroxytryptamine 2A receptor (5-HT2A), encoded by *HTR2A*, is a widely distributed postsynaptic target for serotonin (5-HT) in the human brain. The Genetic Association Database (1) reports 346 unique association studies between single nucleotide polymorphisms (SNPs) in *HTR2A* and human phenotypes. One hundred eighty-three (53%) of these studies find positive genotype–phenotype associations. Most are related to cognition or risk for neuropsychiatric disorders, supporting the presence of functional genetic variants in *HTR2A*, although many other studies fail to find associations (2). *HTR2A* variants also modulate drug response (2), because 5-HT2A is a target for atypical antipsychotics and antidepressants. Despite positive clinical associations and billions of dollars spent annually on drugs modulating 5-HT2A signaling (3), the role of genetic variants remains unclear.

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Two widely studied SNPs in *HTR2A*, rs6311 (-1438G>A) and rs6313 (102C>T), are in high linkage disequilibrium (LD), do not change the encoded protein, and are used interchangeably in genetic association studies. rs6311, located in the *HTR2A* gene promoter, is suspected to yield positive clinical associations via changes in messenger (m)RNA expression. Some studies find significant correlations between rs6311 or rs6313 genotype and mRNA or protein expression (4–6), although other in vivo or ex vivo studies contradict these findings (7–13), perhaps owing to environmental factors that alter *HTR2A* expression (14–18). Specifically, negative results from allelic *HTR2A* mRNA measurements argue against the presence of *cis*-acting SNPs modulating *HTR2A* mRNA expression (7,9). A commonly studied nonsynonymous SNP (rs6314, His452Tyr) unrelated in to mRNA expression is proposed to affect drug-related signaling (19,20) and human memory (2,21). Taken together, a clinical role for any of these variants remains equivocal.

Most disorders implicating *HTR2A* are complex genetic disorders, yet known genetic variants account for only a small portion of the estimated disease risk or treatment outcome, leaving "missing heritability" (22). Full characterization of genetic variants with functional consequences in key risk genes is a critical step toward resolving missing heritability. Testing the hypothesis that *HTR2A* harbors regulatory genetic variants, we surveyed *HTR2A* mRNA expression in human brain, revealing novel alternative exons and untranslated regions (UTRs), which are modulated by common and rare functional SNPs that significantly associate with depression risk and SSRI treatment in the STAR*D study (23,24).

Methods and Materials

Complementary DNA (cDNA) Library Construction

Demographics for postmortem human tissues are listed in Table S1 in Supplement 1. Samples with RNA integrity number

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(RIN) <5 (Agilent 2100 BioAnalyzer analysis, Agilent Technologies, Santa Clara, California) were excluded from study. Seventy-five dorsolateral prefrontal cortex (Brodmann's area [BA]46) samples from different individuals (cocaine abusers and controls) and nine additional brain regions (frontopolar cortex [BA10], Wernicke's Area [BA22], ventral anterior cingulate cortex [BA24], insular cortex, amygdala, hippocampus, putamen, cerebellum, and pontine raphe nuclei) originating from the same individual were used in this study. Sample MB085 was excluded from group expression analyses because it harbors a rare variant significantly affecting mRNA expression, described below. Genomic DNA (gDNA) and total RNA were isolated as previously described (25). cDNA for transcriptome sequencing was reverse transcribed from 10 ng of total RNA using the Ovation RNA-Seq System (NuGEN Technologies, San Carlos, California). For all other ex vivo brain-related experiments, 500 ng of total RNA was primed for reverse transcription with gene-specific primers (Table S2 in Supplement 1) plus oligo-dT using SuperScript III (Life Technologies, Grand Island, New York).

Massively Parallel Sequencing

Ten prefrontal cortex (PFC) BA46 Ovation RNA-Seq libraries were sequenced by SOLiD 4 Next-Generation Sequencing (Life Technologies), and libraries for the nine additional brain regions were sequenced on the 5500 SOLiD System (Life Technologies). Sequenced reads were mapped to National Center for Biotechnology Information Build 37/hg19 of the human genome using LifeScope Genomic Analysis Solutions software (Life Technologies), producing RPKM (reads per kilobase of exon model per million mapped reads) values for normalized mRNA expression. Mapped reads were visualized with the Integrative Genomics Viewer (Broad Institute, Cambridge, Massachusetts [26]).

Quantitative Polymerase Chain Reaction and Allelic mRNA Expression

Gene expression was measured in triplicate in all 74 BA46 PFC gene-specific cDNA libraries via quantitative polymerase chain reaction (qPCR) using primers listed in Table S2 in Supplement 1, as previously described (25). Stepwise linear regression was performed for C_T values, normalized to β-actin (*ACTB*), against *HTR2A* genotypes (Figure 1). Allelic expression imbalance (AEI) was measured, in duplicate for gDNA and triplicate for cDNA, in samples heterozygous for marker SNPs using SNaPshot (Life Technologies) (27). Significant AEI was defined as allelic differences greater than 2 SD of the average within-sample error for the assay.

In Vitro Characterization of rs6311 and 5′UTRs

Five chimeric 5′UTR-luciferase constructs were used for in vitro characterizations (no UTR, short [sUTR], intermediate [mUTR], and long [lUTR] with either rs6311 alleles: lUTR-rs6311/G and lUTR-rs6311/A; Methods in Supplement 1). To measure allelic expression, lUTR-rs6311/G and lUTR-rs6311/A were cotransfected in triplicate (800 ng/well of 12-well cell culture plates) using Lipofectamine 2000 (Life Technologies), along with no transfection controls. Antibiotic-treated media was added after 6 hours to prevent infection. RNA and plasmid DNA was isolated with TRIzol (Life Technologies) by chloroform extraction and precipitation with isopropanol after 48 hours. mRNA AEI ratios were normalized to transfected plasmid DNA allele ratios.

To measure translation efficiency, each of the five 5′UTR luciferase constructs were transfected in duplicate (750 ng/well) using Lipofectamine LTX with Plus Reagent (Life Technologies), including no-transfection controls. After 48 hours, protein and RNA were harvested using the Ambion PARIS system (Life Technologies). For each transfection, equal volumes of purified total cell RNA lysate (10 uL) were used for cDNA synthesis and subsequent quantification of luciferase mRNA expression via qPCR, measured in triplicate. For luciferase protein expression, 25 uL of purified protein lysate and 25 uL of Dual-Glo Luciferase substrate (Promega Corporation, Madison, Wisconsin) were added to a flat-bottom 96-well microplate, and fluorescent intensity was measured in triplicate by a Fusion fluorescent plate reader (PerkinElmer, Waltham, Massachusetts). Fluorescent signal intensity was divided by a transformed C_T value for the corresponding sample, yielding a luciferase protein activity measurement that is normalized to luciferase mRNA expression, reported as percentage of the no UTR construct (Table S3 in Supplement 1).

In Vitro Immunohistochemistry of HTR2A Isoforms

Details are provided in Supplement 1.

CpG Methylation at the HTR2A Locus in Human Prefrontal Cortex

Details are provided in Supplement 1 and Numata *et al.* (28).

Clinical Associations with STAR*D

Details for the STAR*D study are published elsewhere (23,24) (Table S4 in Supplement 1). Covariates and clinical outcomes tested for genetic associations are listed in Supplement 1. Stepwise linear regression or logistic regression was performed for demographic variables against each outcome variable separately and statistically significant covariates were included in

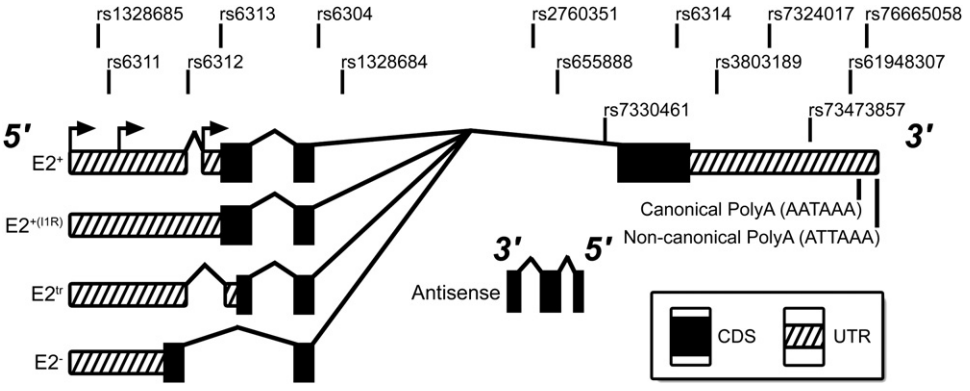

Figure 1. Gene map of the *HTR2A* region and transcripts identified in the current study. Annotated single nucleotide polymorphisms were included in expression quantitative trait locus analyses. Alternative splicing regulates the usage of intron1/exon2 to create four splice isoforms ($E2^+$, $E2^{+(I1R)}$, $E2^{tr}$, and $E2^-$). These transcripts can use multiple transcription start sites (arrows on $E2^+$ 5′ untranslated regions [UTR]) or alternative polyadenylation signals in the 3′UTR. CDS, protein coding DNA sequence.