Supplementary Online Content

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eMethods. Further protocol details

eResults. Further results details

eReferences.

eFigure. Flowchart illustrating the design of the study

This supplementary material has been provided by the authors to give readers additional information about their work.

eMethods

5-HT2AR mRNA expression in post-mortem prefrontal cortex of healthy humans

The publicly available collection used in the present study included post-mortem human brains taken from autopsy primarily from the Offices of the Chief Medical Examiner of the District of Columbia, and of the Commonwealth of Virginia, Northern District, all with informed consent from the legal next of kin (protocol 90-M-0142 approved by the NIMH/NIH Institutional Review Board). Additional post-mortem fetal, infant, child and adolescent brain tissue samples were provided by the National Institute of Child Health and Human Development Brain and Tissue Bank for Developmental Disorders (http://www.BTBank.org) under contracts NO1-HD-4-3368 andNO1-HD-4-3383. The Institutional Review Board of the University of Maryland at Baltimore and the State of Maryland approved the protocol, and the tissue was donated to the NIMH under the terms of a Material Transfer Agreement. Clinical characterization, diagnoses, and macro- and microscopic neuropathological examinations were performed on all CBDB cases using a standardized paradigm. Details of tissue acquisition, handling, processing, dissection, clinical characterization, diagnoses, neuropathological examinations, RNA extraction and quality control measures were described previously[1,2]. The Brain and Tissue Bank cases were handled in a similar fashion (http://medschool.umaryland.edu/BTBank/ProtocolMethods.html). Toxicological analysis was performed on every case. Subjects with evidence of macro- or microscopic neuropathology, drug use, alcohol abuse, or psychiatric illness were excluded.

From each subject in the brain collection, RNA from prefrontal grey matter was analyzed using spotted oligonucleotide microarrays yielding data from 30,176 gene expression probes and allowing us to focus on 5-HT2AR mRNA expression. In particular, total RNA was extracted, amplified and fluorescently labeled. Reference RNA was pooled from all samples and treated identically to sample RNAs. Labeled RNAs were hybridized to two-color custom-spotted arrays from the NHGRI microarray core facility. After normalization[3], $\log_2$ intensity ratios were further adjusted to reduce the impact of known and unknown sources of systematic noise on gene expression measures using surrogate variable analysis[4].

DNA from cerebellar tissue was studied with Illumina BeadChips producing 625,439 SNP genotypes called using the BeadExpress software for each subject[2].

5-HT2AR mRNA and protein expression in HeLa cells

- HTR2A rs6314 C and T cDNA synthesis and cloning in expression vector: The *HTR2A* rs6314 cytosine *and* thymine coding sequences (CDSs) were obtained from 500ng of Ambion® FirstChoice® Human Brain Reference RNA using SuperScript™ One-Step RT-PCR with PlatinumR Taq (Invitrogen). In order to clone the coding sequences (CDSs), RT-PCR on total cellular RNA was performed by annealing primers to the first and the last nucleotides of the complete *HTR2A*. The forward primer (5'- ACG GAT CCG AGA TGG ATA TTC TTT GTG AAG AAA ATA C- 3') contained at the 5' end the BamHI restriction site, the Kozac sequence and the starting codon ATG while the reverse primer (5'- AAG AAT TCT CAC ACA CAG CTC ACC TTT TC - 3') contained the EcoRI site at the 5'end. Amplification products were purified by Nucleo Spin® extract II (Macherey Nagel), cloned into the pGEM-T easy vector (Promega), and transformed into high efficiency Escherichia coli JM109 Competent Cells (Promega). Positive colonies were selected and plasmids were purified by the QIAprep® Spin Miniprep Kit (QIAGEN). All procedures were performed according to the manufacturer's instructions. Purified plasmids were sequenced in order to discriminate clones containing the *HTR2A* rs6314 cytosine and clones containing *HTR2A* rs6314 thymine. *HTR2A* CDSs were subcloned into BamHI/NotI digested pcDNA3 vector (Invitrogen) giving rise to pcDNA3- *HTR2A* rs6314 thymine and pcDNA3-*HTR2A* rs6314cytosin constructs.

- *Transient overexpression of HTR2A rs6314cytosine or thymine alleles in HeLa cells*: HeLa cells (www.lgcpromochem-atcc.com) were grown in DMEM (Dulbecco's modified Eagle's medium, Eurobio, Courtaboeuf, France), implemented with 10% fetal bovine serum and appropriate antibiotics, in 12-well plate at 37°C in a humidified incubator containing 5% CO2 . When the cells were about 60% confluent, the culture medium was replaced by 2 ml of DMEM containing 10% fetal bovine serum without antibiotics and transfected with pcDNA3- *HTR2A* rs6314thymine and pcDNA3-*HTR2A* rs6314cytosin constructs using Satisfaction reagent (Stratagene) according to the manufacturer's instructions. After 24 h, the culture medium containing transfection complexes was removed and replaced by new DMEM with 10% fetal bovine serum and appropriate antibiotics. Cells were harvested and total cellular RNA and total cellular proteins were purified for real time-PCR and western blot analyses, respectively.

- 5-HT2AR *mRNA levels by quantitative Real-time RT- PCR:* Total RNA from HeLa cells was obtained by Aurum ™. Total RNA mini kit and RNA concentration was determined by spectrophotometric measurements at 260, 280, and 230 nm using the NanoDrop® ND 1000 spectrophotometer (ThermoFisher Scientific Inc., MI., Italy). Reverse transcription reaction was performed on 1 µg of total RNA using the RevertAid™ First strand cDNA Synthesis Kit (Fermentas), according to the manufacturer's instructions. The resultant cDNA was used for quantitative, real-time RT PCR . The *β-Actin* gene was used as endogenous control. *β-Actin* and *HTR2A* sequences (GeneBank), were used to build two couples of primers in order to obtain almost the same annealing temperature, using Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and primer3 (http://frodo.wi.mit.edu/) software. Primer sequences were the following: 5' – AGG CAT GCA AGG TGC TGG GC – 3' (*HTR2A*-forward); 5'- ACA CAT TGA GCA GGG CCC CA – 3' (*HTR2A*-reverse): 5'- ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG – 3' (*β-Actin*-forward); 5' – CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC – 3' (*β-Actin*-reverse).

Real time PCR was performed by the ABI Prism 7000 Sequence Detection System (SDS) (Applied Biosystems) using SYBR green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. Thermal cycling conditions included an initial heat-denaturing step at 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 1 min. Following the amplification, melting curves of the PCR products were determined from 70°C to 95 °C to ascertain the specificity of the amplification.

- *Western blotting*: cells were washed twice in cold PBS and then lysed in RIPA buffer (50mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.5 % sodium deoxycholat, 0.1% SDS, protease inhibitors cocktail) for 1h on ice. Lysates were then cleared by centrifugation at 13,000 rpm for 15 min at 4°C, aliquoted and stored at -20°C. The extracted proteins, quantified by the Bradford assay, were dissolved in Laemmli buffer, separated by SDS-PAGE and electroblotted to PVDF membrane. Blot was subsequently blocked in PBS-T (3.2 mM Na2HPO4, 0.5 mM KH2PO4, 1.3 mM KCl, 135 mM NaCl, 0.05% Tween 20, pH 7.4.) and 5% milk powder for 1 h and then incubated O.N. with Anti-HTR2A antibody produced in rabbit (Sigma) at 1:3000 . After 4 ×10 minutes washing in PBS-T, blot was incubated with secondary antibodies, goat anti-rabbit IgG HRP (Santa Cruz Biotechnology) at 1:5000 for 1 h , and chemiluminescence detected by autoradiography with ECL Plus™ from GE Healthcare. The same blot was submerged in stripping buffer (100 mM-mercaptoethanol, 2% (w/v) sodium dodecyl sulfate, 62.5 mM Tris-HCl, pH 6.7) and incubate at 60°C for 30 minutes with occasional agitation. The membrane was washed for 2 ×10 minutes in PBS-T at room temperature and blocked in PBS-T and 5% milk powder for 1 h and then incubated O.N. with a mouse monoclonal anti-actin antibody (Oncogene Research) at 1:2000 and after 4 ×10 minutes washing, the blot was incubated with secondary antibodies, goat anti-mouse IgM HRP (Oncogene Research) at 1:5000, and chemiluminescence detected by autoradiography. Protein quantification was performed by Quantity One software (Bio-Rad).

Genotyping

Genotyping of HTR2A rs6314 polymorphism was performed by TaqMan® based allelic discrimination assay (assay ID: C__11696920_20, Applied Biosystems, Forster City, CA) on a 7900HT Fast Real-Time PCR System platform (Applied Biosystems). Genotype accuracy was assessed by direct sequencing within a subset of 100 samples, showing the same results (PCR and sequencing primers are available on request). The failure rate was <1%. Control samples with known genotype were included in the analysis in order to asses genotyping quality. The agreement rate was >99%.

fMRI tasks and imaging data acquisition and processing

N-back

"N-back" refers to how far back in the sequence of stimuli that the subject had to recall. The stimuli consisted of numbers (1–4) shown in random sequence and displayed at the points of a diamond-shaped box. There was a non-memory-guided control condition (0-back) that presented the same stimuli but simply required subjects to identify the stimulus currently seen. As memory load increased, the task required the recollection of a stimulus seen one stimulus (1-back) or two stimuli (2-back) beforehand while continuing to encode additionally incoming stimuli.

VAC

Each stimulus was composed of arrows of three different sizes pointing either to the right or to the left; small arrows were embedded in medium sized arrows, that were in turn embedded in a large arrow. Subjects were instructed by a cue word (big, medium or small) displayed above each stimulus to press a button corresponding to the direction of the large, medium or small arrows (right or left). To increase the level of attentional control required, the direction of the arrows were congruent or incongruent across all three sizes. This resulted in the following conditions:

- Low level of attentional control (LOW): all three sizes of arrows were congruent in direction with each other. The cue was the word BIG.
- Intermediate level of attentional control (INT). Two stimuli were used: the big arrow was incongruent in direction to the small and the medium arrows in both; the cue was BIG in one of them, SMALL in the other one.
- High level of attentional control (HIGH). Two stimuli were used: the medium size arrows were incongruent in direction to the big and the small arrows in both; the cue was SMALL in one of them, MEDIUM in the other one.
- a simple bold arrow pointing either to the left or right, was used as a sensorimotor condition.

Subjects were instructed to respond to task stimuli with the right hand using a button box (right button for 'right' response, left button for 'left' response), and to press the response button as fast and accurately as possible. Furthermore, they were asked to move their thumb to a small plastic knob placed between buttons after each response. All subjects were trained on the task prior to the fMRI session. Each stimulus was presented for 800 ms, and the order of the stimuli was randomly distributed across the session [5]. The total number of stimuli was 241: 50 HIGH (25 stimuli of each of the two stimulus types that subtended this level of conflict), 68 INT (34 stimuli of each of the two stimulus types that subtended this level of conflict), 57 LOW, and 66 simple bold arrows; the total duration of the task was 10 minutes, 8 seconds. A fixation cross-hair was presented during the interstimulus interval, which ranged from 2000 to 6000 ms.

fMRI data acquisition

We used gradient-echo-planar-imaging sequences, as follows: N-back: 20 contiguous slices; repetition time (TR)/echo time (TE): 2000/30 ms; field of view: 24 cm; matrix: 64x64. VAC: TR/TE=2000/30; 26 interleaved slices, thickness = 4mm, gap = 1mm; voxel size 3.75x3.75x5mm; scans = 300; flip angle = 90°; field of view = 24cm; matrix = 64x64).

Preprocessing of imaging data

Images for each subject were realigned to the first volume in the time series to correct for head motion, spatially normalized into a standard stereotactic space (Montreal Neurological Institute, MNI, template) using a 12 parameter affine model and spatially smoothed (10 mm Gaussian filter). After realignment, data sets were also screened for small motion correction (<2mm in translation, <1.5° in rotation).

For each experimental condition of the N-back task, a box car model convolved with the haemodynamic response function at each voxel was modeled. To account for potential differences in head movement between groups, residual movement was also modeled as a regressor of no interest. Pre-determined condition effects at each voxel were calculated using a t-statistic, producing a statistical image for the contrast of 2-back versus 0-back, 1-back versus 0-back, 0-back versus rest.

fMRI responses during the VAC task were modeled using a canonical hemodynamic response function and temporally filtered using a high-pass filter of 128 Hz and a hrf-shape low-pass filter. Vectors were created for each condition using the timing of correct responses. To account for potential differences in head movement between groups, residual movement was also modeled as a regressor of no interest. A *t* statistic was then used to produce a statistical image for BOLD responses relative to brain processing of stimuli for each level of attentional control (high level - HIGH, intermediate level - INT, and low level - LOW).

Association of fMRI response during N-back and VAC tasks with rs6314 controlling for gray matter volume

Previous studies have demonstrated that rs6314 is associated with Gray Matter (GM) volume, particularly in Medial-Temporal Lobe (MTL) areas, including the hippocampus[6]. Therefore, we investigated whether potential GM differences may have been a confounding factor in the fMRI results[7]. With this aim, Voxel Based Morphometry (VBM) analysis was performed in a sub-sample of the individuals investigated with fMRI (N=175; 158 CC and 19 CT) for whom structural MRI data were also available. Three-dimensional structural MRI images were acquired on the same General Electric (Milwaukee, WI) 3 Tesla scanner using a T1-weighted SPGR sequence (TE=min full; flip angle, 6°; field of view, 250 mm; bandwidth, 31.25; matrix, 256 x 256) with 124 1.3-mm-axial slices). VBM analysis was performed using the VBM8 toolbox[8]. Briefly, the T1-weighted scans were partitioned into different tissue classes — GM, white matter and non-brain voxels (CSF, skull) based on separate tissue probability maps for each tissue class using the "new segmentation" approach in SPM8[9]. In order to compare brains of different subjects, the resulting

segments were normalized to a population template generated from the complete dataset using a diffeomorphic registration algorithm[10]. This new high-dimensional non-linear warping algorithm selects conserved features which are informative for registration, thus minimizing structural variation among subjects and providing optimal inter-subject registration. Subsequently, all images were "modulated" by Jacobian determinants from normalization steps to preserve initial volumes. Following this procedure, images were smoothed by convolution with an isotropic Gaussian kernel of 8 mm full-width at half maximum.

ANCOVA was then used to investigate group-level effects. Orthogonalized first- and second-order polynomials of age, gender, and total GM volume were used as "nuisance" variables, in order to control for any independent effects on our findings and to ensure that the analysis identified regionally specific "non-global" effects[11]. A statistical threshold of $p<0.05$, family wise error small volume corrected for the Brodmann's areas in which significant clusters were located, was used to investigate association with prefrontal gray matter volume. The same threshold, family wise error small volume corrected for hippocampal volume, was used in order to replicate previous findings on association between rs6314 and gray matter in the hippocampus, a brain region of relevance for the pathophysiology of schizophrenia[12].

Despite the absence of robust prefrontal differences in gray matter between the groups (see below), we also assessed the potential relationship between functional and structural data. With this purpose, we built MarsBar ROIs using the clusters associated with the main effect of genotype in the fMRI n-back and VAC analyses as volumes of interest. We then applied these ROIs to the VBM analysis and extracted GM volume values from these prefrontal areas. Thus, we added these GM values as regressors of no interest in new n-back and VAC fMRI ANCOVAs. Of the individuals here studied, 54 healthy subjects for the VAC task (49 CC, 5 T carriers) and 131 healthy subjects for the N-back task (115 CC, 16 T carriers) also had structural MRI data. As in the main ANOVA, we used a statistical threshold of $p<0.05$, minimum cluster size (k)=5, family wise error small volume corrected using as volume of interest the WFU_PickAtlas[13] Brodmann's areas in which significant clusters were located (BA46 for the N-back task, BAs 9 and 46 for the VAC task).

Behavioral tasks

Trail Making Test

The Trail making consists of two versions: Trail Making A and B. Trail Making A requires the connection of consecutive numbers (from 1 to 25) randomly distributed on a card (for e.g., from "1" to "2" to "3" and so on). Trail making B requires the connection of randomly distributed numbers (from 1 to 12) and letters (from A to L in the Italian version) in alternating order (for e.g., from "A" to "1" to "B" to "2" and so on). In both versions, subjects are required to complete the task as quickly as possible without lifting the pencil.

Wisconsin Card Sorting Test

This test uses cards with different forms, colors and numbers. Subjects are required to sort the cards according to different rules (i.e., by color, form, or number). As the test proceeds, there are unannounced rule shifts requiring the subject to modify mental set.

eResults

Bioinformatic modeling of the association between rs6314 and 5-HT2AR expression

At the protein level, histidine to tyrosine amino-acid substitution in cytoplasmic domain revealed medium compatibility according to SIFT (score=0.09), and low compatibility according to PolyPhen (score=2.05). However, prediction on secondary structure did not differ. Further analyses on alignment of homologous sequences revealed aminoacid conservation among Rhesus, Tarsier, Mouse, Dog, Platypus, Chicken, consistent with the hypothesis of medium compatibility.

Association of rs6311 and rs6313 with 5-HTR2A mRNA expression in post-mortem prefrontal cortex

rs6311 and rs6313 have been previously associated with functional effects on *HTR2A* gene function. Therefore, we used the BrainCloud database to seek potential association of these two SNPs with prefrontal mRNA expression in the Caucasian sample used to assess all other associations. ANCOVA, covarying for age at death, post-mortem interval, pH and gender, did not indicate any significant association of these SNPs with post-mortem expression of 5-HT2AR. (rs6311 p=0.7; rs6313 p=0.7). These results further support earlier studies reporting lack of association[14].

Exploratory analysis on association of 5-HTR2A mRNA expression in post-mortem prefrontal cortex with cis- and trans- genetic variants

Using the BrainCloud database, we have performed post-hoc and exploratory analyses to identify other variants in cis- and trans- with potential effects on 5-HTR2A mRNA expression. We analyzed all genotyped SNPs within *HTR2A* in BrainCloud to explore their potential association with post-mortem prefrontal mRNA expression in normal Caucasian individuals[2]. In the BrainCloud database 102 *HTR2A* SNPs have been typed. ANCOVA with age, post-mortem interval, pH, and gender as covariates of no interest indicated lack of statistically significant association for all SNPs (all p>0.1), with the exception of two (rs9567733: p=0.03; rs3803189: p=0.04). Results relative to these two SNPs were obtained collapsing heterozygotes and homozygotes for the minor allele (N=6 and N=2, respectively) in one group. These SNPs are in high LD (D'=1), no previous study has demonstrated any association with HTR2A expression, and they are not in LD with rs6311, rs6313, or rs6314. Importantly, their statistical association with *HTR2A* mRNA expression would not survive Bonferroni correction for the number of SNPs analyzed or Family Wise Error correction, a necessary correction because no prior hypothesis had been made. However, to further control for putative effects of these SNPs on our results, we have investigated the distribution of the relative genotypes in the rs6314 genotype groups. A χ^2 indicates that there is no significant difference in rs9567733 (χ^2=0.14; p=0.7) or rs3803189 (χ^2=0.45; p=0.5) distribution within rs6314 genotypes, suggesting that putative functional effects of these SNPs do not strongly affect our data.

We also interrogated BrainCloud to investigate putative association of genetic variation in trans- with 5-HT2AR post-mortem expression. For this analysis, we used a statistical threshold of $p<10^{-8}$, which is currently used for genome wide association studies. Five SNPs, rs29661401 (chromosome X, intergenic SNP between *BCOR* and *IMPDH1 P2*), rs11924818 (Chromosome 3, Sodium Channel gene – *SCN11A* - intronic), rs17262338 (Chromosome 4, intergenic SNP between *RAPGEF2 and RPS14P7*), rs359688 (Chromosome 2, intergenic SNP between *LOC100422417* and *YWHAZP2*), and rs12200309 (Chromosome 6, intergenic SNP between *FOXQ1 and FOXF2*) survive this threshold. However, χ^2 indicated that the genotypes of these SNPs were not differentially distributed within rs6314 genotype groups (all χ^2<2.4; all p>0.4). Furthermore, most of these SNPs are intergenic variants with unknown biological significance and no reported functional effects on gene function. Moreover, there is no published literature on the putative relationship between these proteins and *HTR2A*. All together, these data suggest that these genetic variants do not strongly affect our results.

Association of fMRI response during the N-back and VAC tasks with rs6314 controlling for gray matter volume

ANCOVA on VBM data (see supplemental methods) indicated a main effect of rs6314 genotype in 3 prefrontal clusters (right BA9, right BA11; left BA6) at the uncorrected statistical threshold of p<0.005. However, these uncorrected results do not survive correction for multiple comparisons within the Brodmann's areas in which these clusters were located. Despite the absence of robust prefrontal differences in gray matter between the groups, we also evaluated whether gray matter volume was in any way associated with the genotype effect on fMRI results. Results of

ANCOVAs covarying for prefrontal gray matter values again indicated for both N-back and VAC task a main effect of rs6314, with T carriers having greater activity relative to CC subjects. These main effects were located in similar clusters and with the same local maxima relative to those found in the analyses without covarying for GM (N-back: x -51 y 38 z 12; Z= 2.9; VAC: x 45 y 26 z 26; Z=4.2). Z values obtained with this analysis are similar to those found with the previous ANOVA (N-back ANOVA Z=3.1; VAC ANOVA Z=4.3).

Replication of the association between rs6314 and gray matter volume in the hippocampus

We also interrogated GM data in order to replicate previous findings on association between rs6314 and volume of the hippocampus[6], a brain region of relevance for the pathophysiology of schizophrenia [12]. Results indicated a main effect of rs6314 in left hippocampus (x -24 y-42 z 0; Z=3.23; $p<0.05$, FWE corrected for hippocampal volume; K=18), with CC individuals having greater GM values relative to CT subjects. These results are consistent with previous findings indicating a similar left lateralized effect in this brain region[6].

Pharmacogenetic study: analysis on potential outliers

Grubbs' test indicated presence of potential outliers when analyzing negative symptoms scores (CC: Grubbs' statistics= 3.3; p=0.05; CT: Grubbs' statistics= 2.9; p=0.002). In particular, two CC and one CT individuals might be considered outliers based on a cut-off value of ± 2 standard deviations from the mean. An ANCOVA excluding these subjects indicates a statistically significant main effect of rs6314, with stronger statistics relative to the previous analysis including the potential outliers (ANCOVA- analysis without outliers: F=9.1; p=0.003; analysis with outliers: F=4.6: p=0.03: χ^2 - analysis without outliers: χ^2: 7.5; p= 0.006; analysis with outliers: χ^2= 6.2; p=0.01).

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eFigure. Flowchart illustrating the design of the study