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A Rapid New Assay to Detect RNA Editing Reveals Antipsychotic-Induced Changes in Serotonin-2C Transcripts

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ABSTRACT

We report the development of a new assay as an alternative to direct DNA sequencing to measure RNA-edited variation in tissue. The new assay has been validated and is accurate, cheaper, more rapid, and less labor-intensive than DNA sequencing. We also outline the statistical modeling required for analyses of the hierarchical, clustered RNA-editing data generated in these studies. Using the new technique, we analyzed the effects of long-term antipsychotic medication on serotonin-2C receptor (5-HT_{2C}R) RNA editing in rat brain. Our hypothesis that a drug with high affinity for 5-HT_{2C}R, such as clozapine, would alter its RNA-editing profile was not confirmed. Whereas haloperidol, a typical antipsychotic drug that is primarily a dopamine receptor antagonist, reduced 5-HT_{2C} VNV isoform frequency and the level of RNA editing at the D site, risperidone and not the prototype atypical antipsychotic drug clozapine increased the frequency of 5-HT_{2C} VNV and D-site editing. Our data emphasize that caution is required in the interpretation of RNA-editing data in studies of psychiatric disorders, because these studies usually include subjects who received long-term exposure to medication. This newly established method will facilitate high-throughput investigations of RNA editing in disease pathology and in the pharmacological activity of drugs.

The revelation that the human genome comprises between 30,000 and 40,000 protein-coding genes was unexpected, because such few genes are unlikely to explain the functional diversity between humans and less complex organisms. These interspecies differences could arise from post-transcriptional processes such as RNA editing and alternative splicing, which allow a single gene to generate several protein variants. The current study focuses on RNA editing. The recent detection of 1637 potential new substrates for RNA editing (Levanon et al., 2004) indicates that it is likely to be more widespread in the transcriptome than originally believed. RNA editing occurs in the pre-mRNA and has many forms, including insertion, deletion, and the conversion of cytidine to uridine or adenine to inosine. In the latter case, editing of specific adenine residues involves their hydrolytic deamination by a double-stranded RNA adenosine deaminase (ADAR) enzyme. Because inosine base pairs with cytidine in transfer RNA, it is predicted to be translated as guanidine by the ribosome. These changes alter the codon and often the amino acid sequence of the protein, usually with functional consequences (Gott, 2003).

Because of their relatively recent discovery, substrates of RNA editing have not been extensively characterized. Adenine-to-inosine RNA editing in mammalian brain has been identified in ionotropic receptors (such as the GluR2 subunit of the α-amino-3-hydroxy-5-methylisoxazole-4-propionate receptor) and in the serotonin (5-HT)-2C receptor (R), which is coupled to GTP-binding protein. Discrepancies between genomic DNA and cDNA sequences led to the discovery of nucleotide changes caused by the activity of ADAR enzymes. In the fully edited 5-HT_{2C}R, three amino acid codons in the pre-mRNA are changed so that the sequence coding for IRNPI becomes VRGPV (Fig. 1) (Burns et al., 1997).

RNA editing produces many coexisting, functionally dis-

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ABBREVIATIONS: 5-HT_{2C}R, 5-HT_{2C} receptor; ADAR, adenosine deaminase acting on RNA; PCR, polymerase chain reaction; RT-PCR, reverse-transcription polymerase chain reaction; ICC, intraclass correlation coefficient; GEE, generalized estimating equation; OR, odds ratio; bp, base pair; CI, confidence interval.

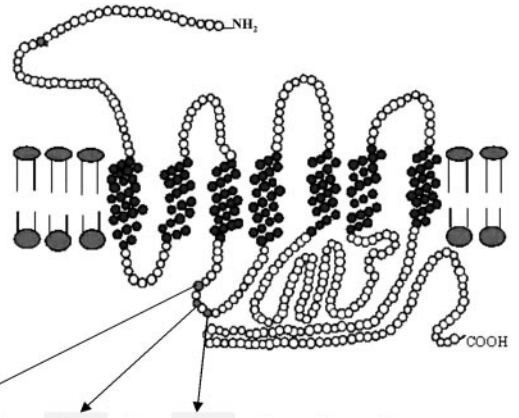
tinct variants of 5-HT_{2C}R. RNA extracted from tissue expressing the 5-HT_{2C} gene is a heterogeneous mixture of up to 32 different 5-HT_{2C} mRNA transcripts. These are predicted to encode 24 protein isoforms and have been detected in both rodent and human brain, with regional differences in their frequencies (Burns et al., 1997; Niswender et al., 2001). Pharmacological evidence shows that 5-HT_{2C} RNA editing reduces constitutive activity and G-protein activation in response to agonist stimulation (Niswender et al., 1999). Therefore, the RNA editing process represents a regulatory mechanism by which cells can modulate their response to environmental stimuli by altering the efficacy and specificity of G-protein to receptor interactions. It is possible that disruption of this process could have pathophysiological effects, but this has not been extensively explored because RNA editing is difficult to detect and quantify.

Limited data indicate that altered RNA editing is associated with a variety of disease processes. Most notably, reduced RNA editing of GluR2 has been found in spinal motor neurons of patients with amylotrophic lateral sclerosis (Kawahara et al., 2004). Furthermore, altered levels of RNA-editing enzymes have been detected in inflamed tissues (Yang et al., 2003) and in malignant gliomas (Maas et al., 2001), whereas studies of psychiatric disease have revealed altered 5-HT_{2C} RNA-editing patterns in schizophrenia (Sodhi et al., 2001) and in suicide (Niswender et al., 2001; Gurevich et al., 2002). However, there are conflicting data (Dracheva et al., 2003), and whether the observed changes in subjects treated with antidepressants are caused by drug treatment is unresolved (Gurevich et al., 2002). More extensive investigations are needed to clarify these findings. Until now, this has been a daunting task, because the complete assessment of RNA editing has involved extensive DNA purification and sequencing, impeding replication studies in the large number of subjects needed for optimum statistical power (see *Results*). Therefore a high-throughput RNA-editing detection method would be invaluable.

Herein, we present a newly developed assay for RNA editing modeled on one of the more complex substrates, the multiply edited 5-HT_{2C} pre-mRNA. The new RNA-editing detection assay is as accurate and informative as DNA sequencing. It is much cheaper and can be adapted for high-throughput protocols and for other RNA-edited substrates. Using this assay, we tested the effects of antipsychotic treatments on 5-HT_{2C} mRNA editing. Furthermore, we clarify the hierarchical sampling design of RNA-editing data sets, investigate the impact of clustering on statistical power analyses, and guide statistical modeling choices appropriate for future studies of RNA editing.

Materials and Methods

Preparation of 5-HT_{2C} cDNA Template. Messenger RNA (2 μg) was extracted by standard methods and pretreated at 37°C for 30 min with 1 unit of RQ-1 RNase-free DNase and 10 units of RNasin RNase inhibitor (both from Promega, Madison, WI), followed by 6 min at 70°C. Reverse transcription was carried out using 200 units of Moloney murine leukemia virus reverse transcriptase with 10 units of RNase inhibitor (Promega), 30 ng of poly(dT) oligonucleotides (Oswel, Southampton, UK), buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, and 10 mM dithiothreitol), and a 0.5 mM concentration of each dNTP (Promega). The reaction mixture was incubated at 42°C for 1 h followed by 72°C for 10 min. The cDNA produced was



Amino acid sequence if 'INI', (unedited)	Tyr	Val	Ala	Ile	Arg	Asn	Pro	Ile	Glx	His	Ser
5-HT_{2C} INI or genomic DNA	TAT	GTA	GCA	ATA	CGT	AAT	CCT	ATT	GAG	CAT	AGC
Amino acid sequence if 'VSV'				Val		Ser		Val			
5-HT_{2C} VSV	TAT	GTA	GCA	GTG	CGT	AGT	CCT	GTT	GAG	CAT	AGC
Amino acid sequence if 'VGV'				Val		Gly		Val			
5-HT_{2C} VGV	TAT	GTA	GCA	GTG	CGT	GGT	CCT	GTT	GAG	CAT	AGC
Amino acid sequence if 'VNV'				Val		Asn		Val			
5-HT_{2C} VNV	TAT	GTA	GCA	GTG	CGT	AAT	CCT	GTT	GAG	CAT	AGC
RNA EDIT SITES				A B		EC		D			

Fig. 1. Sequence changes observed in the 5-HT_{2C} RNA edited region. The sites of A to I editing, detected as "A" to "G" changes, are indicated as underlined Gs. The top sequence represents the genomic DNA sequence, which is also detected in the cDNA when RNA editing has not occurred (i.e., in the 5-HT_{2C} INI unedited isoform). The sequence of one of the frequently edited isoforms, 5-HT_{2C} VSV, has "A" to "G" changes at sites A–D, which alter three amino acids. The 5-HT_{2C} VGV fully edited isoform has "A" to "G" changes at sites A–E, leading to three altered amino acids. 5-HT_{2C} VNV, 5-HT_{2C} VSV, and 5-HT_{2C} VNI isoforms were found to be frequent in this study.

diluted with nine parts of sterile deionized water. The subsequent PCR amplification of the 5-HT_{2C} RNA-edited region was performed using oligonucleotide primers designed to span the rat exon 3/intron 3 boundary to prevent the amplification of residual contaminant genomic DNA. This PCR reaction included template cDNA (90% dilution), 1 mM concentration of each primer MSF (5′-TTTCAACTGCGTCCATCATGCACCT-3′) and MSR (5′-AACGAAGTTGGGGTCATTGAGCAC-3′), 250 mM concentration each of dATP, dCTP, dGTP, and dTTP (dNTPs), 2 units of DNA polymerase and recommended buffer (Qiagen, Valencia, CA) in a total volume of 20 μl. Samples were amplified for 27 cycles (in the linear phase of amplification). Cycling conditions comprised 1 cycle at 96°C for 5 min followed by 27 cycles at 96°C for 15 s, 50°C for 30 s, and 72°C for 30 s, and finally 1 cycle of 72°C for 5 min in a thermal cycler (Peltier; MJ Research, Watertown, MA), resulting in a 236-bp product. DNA fragments were resolved by 1% agarose gel electrophoresis, stained with ethidium bromide (50 ng/ml), excised from the gel, and purified using Qiaex II (Qiagen).

Characterization of RNA Edited Transcripts. The gel-purified DNA was ligated and cloned using the pGEM-T system II (Promega). Randomly picked colonies were either characterized using direct sequencing or were identified using the new pyrosequencing method (Fig. 2). The DNA sequencing protocol required overnight subcloning, and the DNA was purified and sequenced according to the automated ABI protocol using an ABI377 sequencer (Applied Biosystems, Warrington, UK).

In contrast, with the new pyrosequencing protocol, the liquid culture and DNA purification steps were not necessary. Instead, bacteria from each colony were directly inoculated in a PCR reaction mixture containing 0.2 μM sense oligonucleotide primer (5′-ATATCGCTGGATCGGTATGTAG-3′) and 0.2 μM antisense biotinylated primer (5′-BIOTIN CGAATTGAAACGGCTATGCT-3′), 1× Taq Gold buffer with 1.5 mM $MgCl_2$ (PerkinElmer Life and Analytical Sciences, Boston, MA), 0.75 units of Taq Gold (PerkinElmer), and 0.24 mM dNTPs. Samples were heated to 96°C for 5 min and then amplified for 45 cycles each consisting of 20 s at 96°C, 30 s at 58°C, and 20 s at 72°C in a thermal cycler (Peltier; MJ Research), resulting in a 60-bp product in 30 μl of total reaction volume. A total of 5 μl was resolved by 3% agarose gel electrophoresis, with a 20-bp DNA standard (Qiagen) to ensure successful transformation. The remainder of PCR product was used for the pyrosequencing assay.

Pyrosequencing is a nonelectrophorectic method of DNA synthesis that uses a luciferase-based enzyme reaction to monitor DNA synthesis in real time and is widely used for genotyping (Fakhrai-Rad et al., 2002). A pyrosequencing assay was adapted for use as an alternative to direct DNA sequencing for the identification of RNA edited isoforms. PCR product (25 μl) was mixed with 4 μl of streptavidin-coated Sepharose beads (Amersham Biosciences, Uppsala, Sweden) and 21 μl of binding buffer (10 mM Tris-acetate, 2 M NaCl, 1 mM EDTA, and 0.1% Tween 20, pH 7.6). The mixture was agitated at 1400 rpm for 10 min at room temperature and transferred to a MilliQ filter plate (Millipore, Molshelm, France). The plate was affixed to a vacuum, and liquid was removed. A 50-μl sample of 0.2 M NaOH was added to the plate, incubated for 1 min, and removed by vacuum. Next, 150 μl of 10 mM Tris-acetate, pH 7.6, was applied twice and removed immediately as before. A 54-μl sample of annealing buffer (20 mM Tris-acetate and 2 mM Mg-acetate, pH 7.6) was mixed with 1.92 μl of 10 μM sense primer (5′-ATATCGCTGGATCGGTATGTAG-3′) and used to resuspend the immobilized template in the filter plate. Thereafter, 45 μl of this mixture was transfered to a 96-well PSQ plate (Pyrosequencing, Uppsala, Sweden). The plate was covered, and the annealing reaction proceeded in a thermal cycler (MJ Research) at 80°C for 2 min. Enzyme and substrate reagents from the sodium nitroprusside reagent kit 5 × 96 (Pyrosequencing) were dissolved in 620 μl of high-purity water after reaching room temperature and loaded onto a PSQ 96 reagent cartridge (Pyrosequencing). Of each dNTP, 200 μl was also loaded into compartments in the PSQ 96-reagent cartridge, as instructed by the manufacturer's protocol. The sample plate and cartridge were inserted into the $PSQ^{HA}96$ instrument, and analysis occurred automatically. The dispensation sequence entered into the $PSQ^{HA}96$ analysis program was GCAGCTAGTCGTAGAGTCTAGCT. The Biotage.com technical support team (http://www.biotage.com/) designs dispensation sequences using proprietary software on request. PCR contamination was eliminated by fastidious PCR procedures, including the use of a filtered PCR cabinet, "DNA away" reagent (Molecular BioProducts, San Diego, CA), filtered pipette tips and regular UV irradiation of all equipment. If present, contamination can be detected in negative controls or "blanks" and by the presence of extra peaks, indicating the presence of both G and A at a single site in a transcript. The presence of any apparent "heterozygosity" indicated contamination, thus ensuring the stringency of the method.

Animals and Drug Treatments. Male Sprague-Dawley rats (Harlan, Olac, UK) weighing 250 to 300 g received once-daily injec-

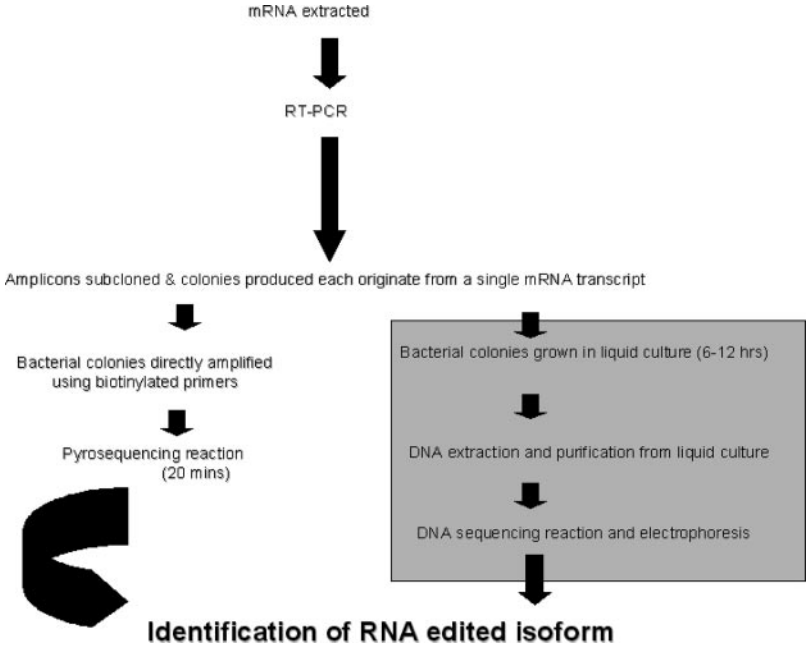


Fig. 2. A direct comparison between the new RNA-editing assay and the direct sequencing protocol. RNA is extracted by standard methods, and RT-PCR is performed to produce a mixed population of products, with varying degrees of RNA editing. The products can be analyzed by subcloning and then either sequencing or pyrosequencing. RT-PCR products are subcloned, and colonies are chosen at random and tested for insert using PCR. Each colony represents one mRNA transcript within the heterogeneous population of mRNAs. For DNA sequencing (represented on the right side of the chart), positive colonies are incubated overnight in liquid culture, and DNA is extracted and purified before sequencing can be performed. The left side of the flowchart illustrates the steps involved in the new assay. The steps eliminated from the direct sequencing method are contained by the box on the right side of the flow chart. Therefore, the new assay eliminates the need for overnight bacterial culture, DNA extraction, purification, and sequencing, which considerably reduces time and expense, thereby facilitating the high-throughput processing of samples.

tions intraperitoneally for 14 days with clozapine (25 mg/kg), haloperidol (1 mg/kg), risperidone (0.5 mg/kg), chlorpromazine (15 mg/kg), or saline vehicle. Animals were killed 6 h after the final dose by a lethal injection of pentobarbital and perfused transcardially with phosphate-buffered saline. Brains were removed, frozen on dry ice/alcohol slurry, and stored at −80°C. All procedures were carried out in accordance with United Kingdom ethical and legal regulations. Cryotome sections (18 μm) were cut from each block (corresponding coronal brain sections at the level of the hippocampus), and 12 sections were homogenized before RNA extraction.

Statistical Analysis: Statistical Power and Data Clustering. To date, inadequate attention has been given to appropriate statistical analysis of RNA-editing measures. In these experiments, the data are usually collected as multiple categorical measures (e.g., isoform type) per subject. Repeated measures from the same subject may not be independent. Greater similarity in the measures within a subject compared with measures in the rest of its group, is defined as data "clustering". Failure to recognize the presence of clustering in data can produce misleading standard errors, confidence intervals, and probability values. The degree of data clustering and cluster size (RNA molecules processed per animal) are critical, because clustering of observations within animals reduces statistical power. The presence and strength of clustering can be quantified by the intraclass correlation coefficient (ICC; the ratio of between-cluster variation to total variation).

If the presence of significant data clustering is confirmed by the ICC, additional observations must be added to the sample size needed if clustering was absent (ICC = 0). The effect of sample clustering on sample size calculations is quantified by the design effect (Snijders and Bosker, 1999): design effect = $1 + (n' - 1) \times ICC$, where ICC is the intraclass correlation and n' is the average cluster size. The following formula shows that the design effect increases with cluster size and ICC. Figure 3 illustrates the effect of different degrees of data clustering on statistical power achieved with different sample sizes (numbers of subjects and clones) considering large, medium, and small effect sizes. For example, if the ICC is 0.10 and there are 10 measures per animal, the design effect is $1 + (10 - 1) \times 0.10 = 1.9$. This means that to have the same power as an unclustered analysis (with ICC = 0), almost twice the number of observations would be needed. If we needed "N" animals without clustering and then discovered that ICC > 0, the larger number of animals required would be the following: number of clusters = N × (Design Effect) = $N \times ([1 + (n' - 1) \times ICC])$, where n' = average cluster size in observations per animal.

Therefore if we use five animals each with 10 clones for a total of 50 observations, and the ICC is 0.1, we would need $5 \times (1 + (10 - 1) \times 0.1) = 5 \times 1.9 = 9.5$ (i.e., 9 or 10 animals). An ICC value of 0.1 may be considered medium clustering (Raudenbush and Liu, 2000), and with 10 observations per animal, a moderate amount of clustering almost doubles the necessary number of measurements to achieve the same statistical power.

Accurate estimation of statistical power must consider the possibility of clustered data. Traditional power analysis (Cohen, 1988, 1992) can estimate the required sample size in the absence of clustering, after which the design effect and corrected number of animals or patients can be calculated. If no pilot data are available to estimate an ICC, one may plot a range of cluster sizes and ICCs, as shown in Fig. 3.

Statistical Analysis: Comparisons of Drug Treatments. Appropriate analyses of data that are significantly clustered include 1) deriving a summary statistic for each subject (e.g., level of editing at each site) and comparing the distribution of summary statistics; 2) using a robust estimate of variance that corrects for the effect of clustering on the standard error calculation; 3) using a random-effects model, that explicitly models the similarity of units within clusters; and 4) using a generalized estimating equation (GEE) that adjusts both standard errors and parameter estimates to allow for clustering (Kirkwood and Sterne, 2003a). GEE was used in the analyses of data generated in the current study.

We applied GEE models to clustered binary-dependent variables, a modeling framework that has not been used in previous studies of 5-HT_{2C} RNA editing. We modeled the effects of drug treatment on each dependent variable (either a predicted 5-HT_{2C} protein variant or a single site of RNA editing) using a GEE model with binomial family, logit link, and exchangeable correlation. In these models, saline was the reference category or odds ratio denominator (saline odds ratio was considered to be 1.0). We described the data using binary variables, including presence or absence of specific 5-HT_{2C} protein isoforms and presence or absence of editing at specific sites A to E. The level of RNA editing at specific sites was calculated as the percentage edited fraction of the total number of molecules processed for that subject. To determine whether antipsychotic drug treatments alter 5-HT_{2C} RNA editing, we sampled a mean of 15 clones from each of 31 Sprague-Dawley rats (470 clones in total) and randomized to one of four antipsychotic treatments or a saline control treatment (see *Animals and Drug Treatments*). We tested for RNA editing differences between saline and drug treatment conditions by measuring protein isoform or RNA edit site frequencies. Protein isoform or RNA edit site categories with less than 5% frequency were not analyzed. The probability of detecting a false positive effect (α) was considered to be 0.05. Before using the GEE models, we tested for the presence of data clustering in the dependent variables using a likelihood ratio test.

Results

Until now, the most complete information of RNA-editing levels and frequencies of individual isoforms has been obtained by direct DNA sequencing of cloned RT-PCR products. Therefore, validation of the new high-throughput method has been performed using DNA sequencing. Substitution of DNA sequencing with the new assay reduces sample processing time from days to hours and reduces the cost of reagents to less than 10% of the usual price of DNA purification and sequencing.

Validation of the New Method. The key to the new method is the identification of RNA-edited transcripts by adapting a technique used for genotyping DNA, pyrosequencing. In our protocol, RT-PCR of the 5-HT_{2C} RNA-edited region is required, and subsequently, the sampled amplicons are subcloned and single colonies (each of which corresponds to an individual transcript) are amplified directly to produce a template for identification using a pyrosequencing assay. The protocol is described in detail under *Materials and Methods* and is summarized in Fig. 2.

The new assay was validated using sequenced plasmid DNA. Eleven sequenced clones were identified with 100% accuracy when both sense and reverse strands were analyzed by the new assay. Figure 4 displays the results of sequencing analyses and pyrosequencing identification of each clone, which were in complete agreement.

5-HT_{2C} RNA Editing in Rat Brain after Long-Term Antipsychotic Treatment. Application of the novel high-throughput 5-HT_{2C} RNA-editing assay in an original data set exhibits the usefulness of this assay and reveals that RNA editing is altered by antipsychotics. Haloperidol (1 mg/kg) and risperidone (0.5 mg/kg) significantly altered 5-HT_{2C} RNA editing in rats treated with a 2-week regimen of daily injections.

Haloperidol decreased the level of editing at the D site [odds ratio (OR) = 0.37, p = 0.009, 95% CI = 0.17 to 0.78],

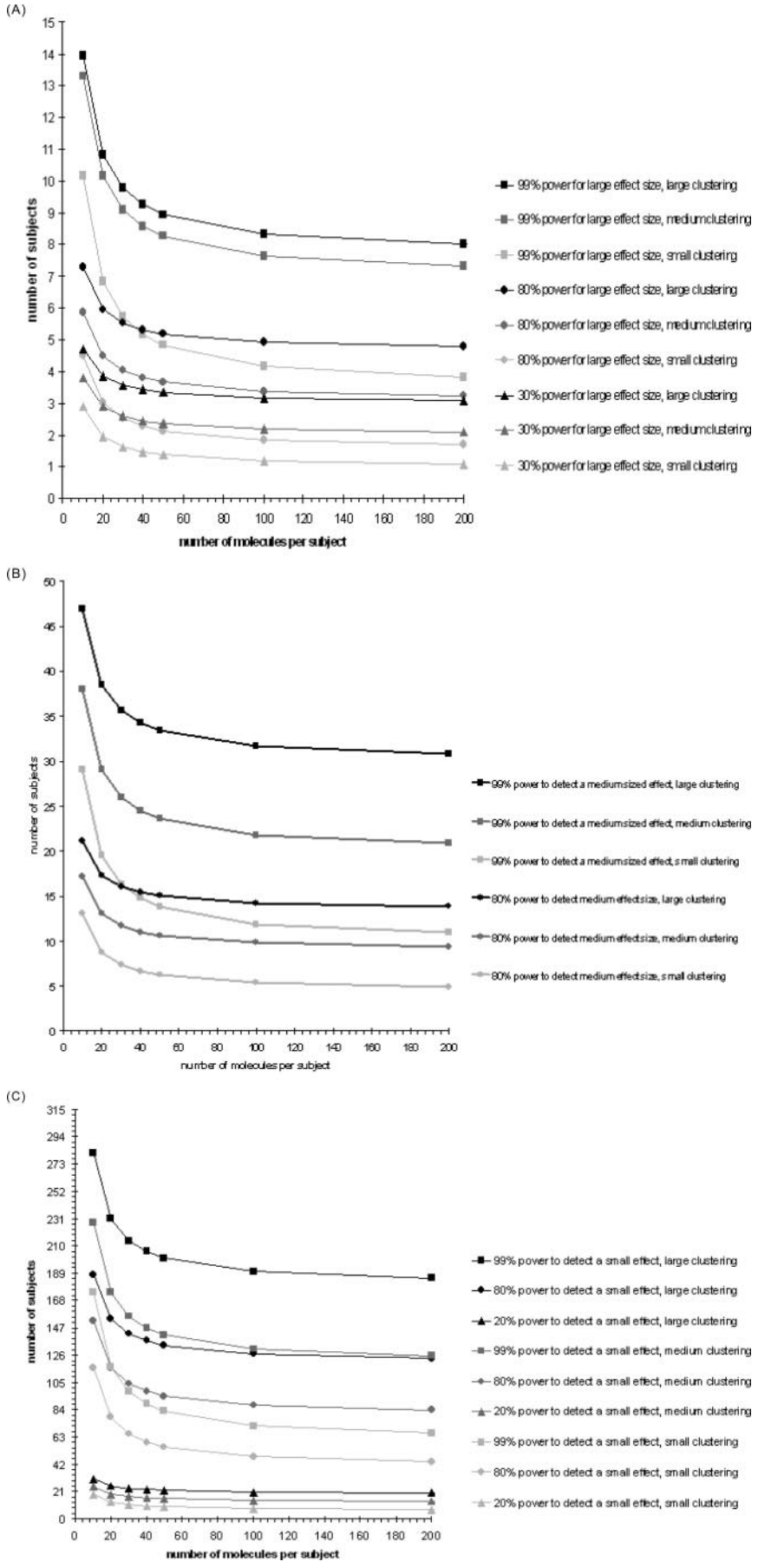


Fig. 3. Graphs to illustrate sample sizes required to detect small (A), medium (B), or large (C) effect sizes. The number of subjects and molecules processed per subject are compared at different levels of data clustering and statistical power. These graphs show that increased data clustering reduces statistical power, and therefore estimation of cluster size is especially critical if medium or small effect sizes are being measured.

leading to increased 5-HT_{2C} VNI isoform frequency (OR = 3.07, $p = 0.017$, 95% CI = 1.22 to 7.71) and decreased 5-HT_{2C} VNV isoform frequency (OR = 0.48, $p = 0.030$, 95% CI = 0.25 to 0.93). By contrast, risperidone increased 5-HT_{2C} VNV isoform frequency (OR = 3.05, $p = 0.005$, 95% CI = 1.40 to 6.65). Risperidone also tended to alter editing levels at the B, C, and D sites, although the direction of this effect relative to saline was different at each site, and these differences were less marked ($p < 0.10$). Neither clozapine nor chlorpromazine treatment led to statistically significant changes in any 5-HT_{2C} RNA-editing variable analyzed. Results are summarized graphically in Fig. 5.

RNA editing measurements with less than 5% frequency were excluded from the analysis (see *Materials and Methods*). Three 5-HT_{2C} protein isoforms had greater than 5% frequency: 5-HT_{2C} VNI, 63/470; 5-HT_{2C} VNV, 224/470; and 5-HT_{2C} VSV, 112/470; therefore, these were included. When editing at individual sites was considered, only the unedited A and edited E sites were too infrequent to analyze. B-site editing levels were significantly clustered by animal after accounting for the treatment effect (ICC = 0.11, 95% CI = 0.03 to 0.32, $\chi^2 = 7.01$, $p = 0.004$). Of six GEE models tested (Fig. 5), the full model probability was significant for the D site ($p = 0.0025$) and 5-HT_{2C} VNI ($p = 0.0192$) and 5-HT_{2C} VNV ($p = 0.0001$) isoforms using Wald tests (4 df).

Analysis of Data Clustering. We demonstrate data analysis in the current study by considering RNA editing at the B site, which was measured as either edited or unedited. Effect size for a χ^2 test was considered to be Cohen's w. Our aim is for sufficient statistical power to detect small or medium effects, defined as $w = 0.1$ and 0.3, respectively (Cohen, 1992), and the probability of detecting a false-positive effect (α) was considered to be 0.05. We tested 31 animals (four treatment groups of six, and one group of seven animals), with 15 clones sampled per animal ($31 \times 15 = 465$ clones). The ICC value was calculated to be 0.11. The effects of saline treatment on binary RNA-editing measures were compared separately with the effects of each drug treatment in a 2×2 χ^2 table (1 df), with 7 versus 7 animals and 15 clones per animal, for a total of 210 measures of presence or absence of an edited B site in each group. If we ignore clustering and assume that the measures are independent (ICC = 0), we estimate 99% power to detect a large and medium effect sizes but only 30% power to detect a small effect. However, the observations from each animal are not independent in this case, and we need to consider clustering when estimating statistical power. The design effect was calculated to be $1 + (n' - 1) \times ICC = 1 + (15 - 1) \times 0.11 = 2.54$. The number of clusters (or subjects) actually required for the statistical power calculated, when the ICC is really 0.11, is n/n' $[1 + (n' - 1) \times ICC] = 210/15 \times 2.54 = 35.6$ animals. The actual number of clones required for the sample power to be as predicted above is $35.6 \times 15 = 533$ (i.e., more than twice the number sampled). Dividing by the design effect suggests that the sample of 210 clones has the statistical power of a sample size of 83 independently sampled clones (210/2.54 = 83). Standard power tables indicate that the genuine power to detect large effect sizes is 99%, for medium effects there is 77% power, and there is only 15% power to detect a small effect. Using Cohen's traditional criterion of 80% as adequate power, we have adequate power only to detect large effects; if the effects are medium or small, the study is underpowered to detect changes in B-site editing.

Discussion

Development of the Novel Assay. We report a simple but powerful new method of facilitating the evaluation of RNA editing. Several genotyping strategies were attempted previously as alternatives to DNA sequencing to identify cloned transcripts (e.g., dot blotting and restriction fragment length polymorphism), but only our adapted pyrosequencing protocol produced accurate reproducible data with a straightforward procedure. Our pyrosequencing assay was validated in comparison with DNA sequence data and was found to be 100% accurate on both strands. The new method, which eliminates the need for overnight liquid culture and subsequent plasmid DNA extraction and purification stages, is therefore less labor-intensive and 90% less expensive than direct DNA sequencing. Hence, our new assay has replaced the use of primer extension and direct DNA sequencing in our research group.

Direct sequencing has been the preferred method in all the detailed studies of 5-HT_{2C}RNA editing to date because it is the only method currently available for determining 5-HT_{2C} isoform profiles (Sodhi et al., 2001; Gurevich et al., 2002; Dracheva et al., 2003; Yang et al., 2004). In addition, direct sequencing has been used for the identification of multiple RNA-editing sites produced by overexpression and subsequent hyperediting activity of the RNA editing enzyme APO-

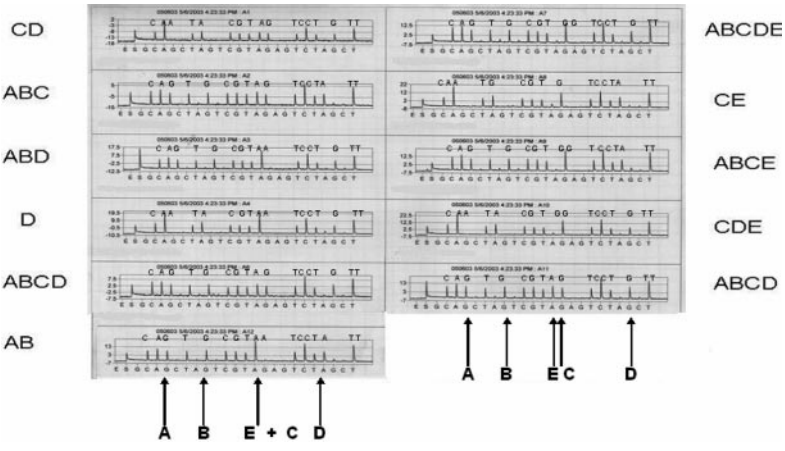


Fig. 4. Validation of the new high-throughput RNA-editing assay. Cloned plasmid DNAs from the 5-HT_{2C}R RNA edited region were first sequenced using standard DNA sequencing techniques and subsequently processed using the new high-throughput assay. The pyrograms demonstrate the accuracy of the new assay in the identification of each RNA-edited site. Each trace is labeled according to the sequencing data, which were replicated by the new method in every clone tested. The positions of sites vulnerable to RNA editing are indicated, and each peak is labeled to provide the clone sequence.

BEC (Yamanaka et al., 1996) or for the identification of 16 naturally occurring edited sites in ADAR 2 (Dawson et al., 2004). The assay developed in this study can be adapted to quantify changes in these and other newly discovered RNA edited substrates. Although our protocol is optimized using pyrosequencing technology, similar DNA synthesis genotyping platforms have been developed by GenoVoxx (Luebeck, Germany) and 454 Life Sciences (Branford, CT), which could be adapted for the assessment of RNA editing with minimal alterations to the current protocol.

Effects of Antipsychotic Drugs on 5-HT$_{2C}$ RNA Editing. This is the first report of altered 5-HT$_{2C}$ RNA editing after long-term treatment with antipsychotic drugs. Haloperidol, a commonly prescribed antipsychotic drug, reduces D-site editing, thereby increasing the frequency of the 5-HT$_{2C}$ VNI isoform and reducing the frequency of the 5-HT$_{2C}$ VNV isoform. Risperidone had the opposite effect. It is not clear why haloperidol, an antipsychotic drug with no affinity for 5-HT$_{2C}$R, would alter its RNA editing. Because chlorpromazine did not duplicate these effects, it seems that the common property of dopamine receptor antagonism is unlikely to be the mechanism. Although the decrease in RNA editing may be related to a generalized toxic effect of haloperidol, this is improbable because the reduction was not uniform across editing sites. Further studies including specific ligands for the receptors targeted by risperidone and haloperidol are required to determine which receptor(s) are involved in the mechanism producing the RNA editing changes observed. Clozapine did not cause any significant change in RNA editing; although our study had sufficient power (80%) to detect medium-sized effects (with the exception of changes at the B site, see *Analysis of Data Clustering* under *Materials and Methods*), it is possible that small effects were missed.

Altered D-site editing of the 5-HT$_{2C}$R is responsible for the

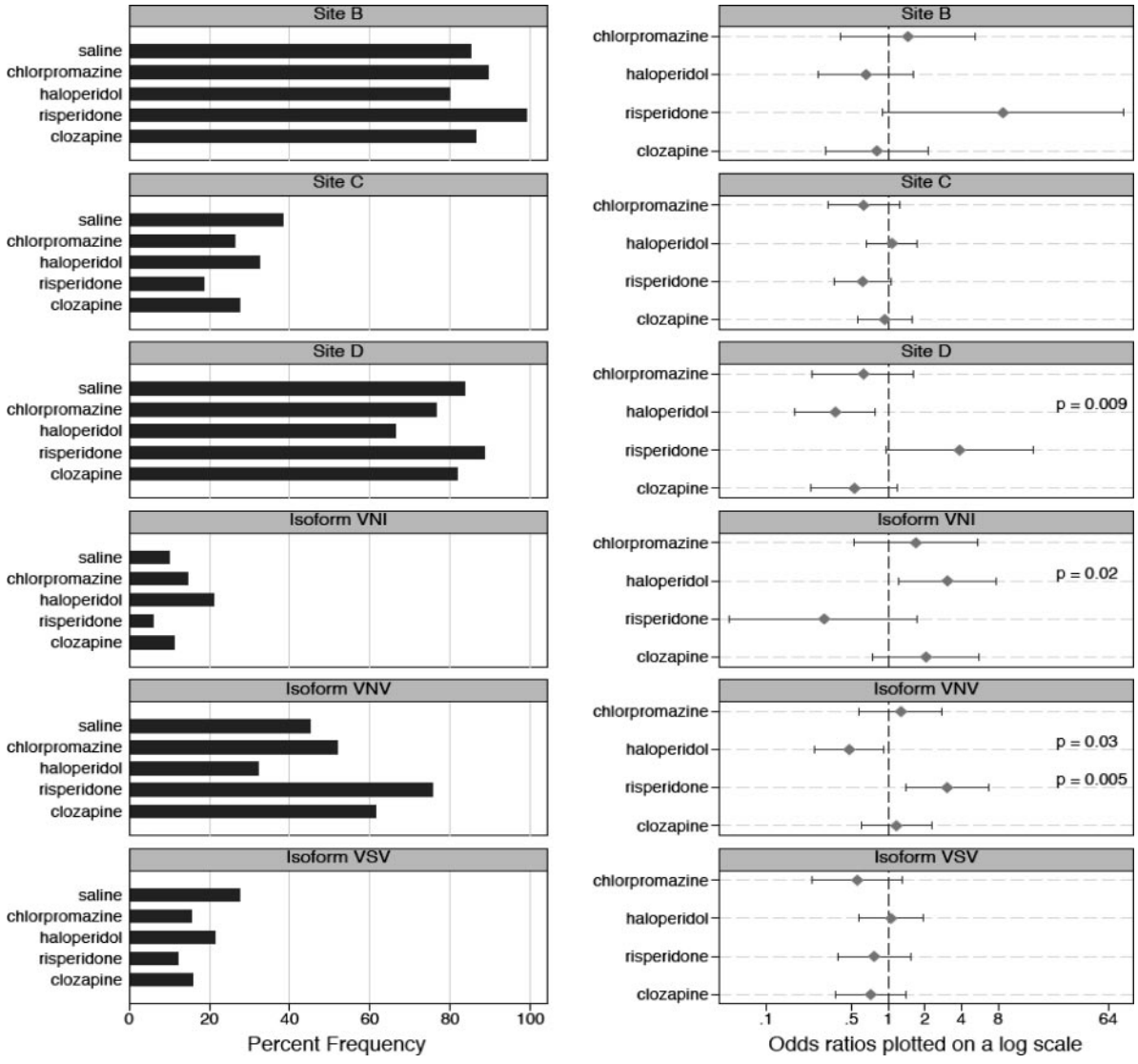


Fig. 5. The effects of antipsychotic drug treatments on 5-HT$_{2C}$ RNA editing in rat brain. The bar chart on the left illustrates the percentage frequencies of each variable measured in the different treatment groups. Saline is the reference category for all models, B, C, and D are the percentage of editing levels. VNI, VNV, and VSV are the inferred 5-HT$_{2C}$-edited isoforms. The average measurements of each variable for the saline-treated group of animals can be compared directly with each drug-treatment group. The chart on the right illustrates the point OR values generated by GEE for each drug treatment group plotted on a logarithmic odds ratio scale (with the horizontal axis labeled on the OR scale). The 95% confidence intervals are indicated as bars around the point OR. The saline treatment is considered to have a point OR = 1. The statistical significance of the most robust changes is indicated.

change in 5-HT_{2C} VNI and 5-HT_{2C} VNV isoform frequencies. Because isoforms differing at the D site have similar G-protein coupling and constitutive activities in vitro (Herrick-Davis et al., 1999), it is reasonable to assume the editing changes resulting from haloperidol or risperidone treatment will be functionally silent, at least with respect to these components of 5-HT_{2C}R activity. The D site of 5-HT_{2C}R is edited by ADAR 2 (Burns et al., 1997), and therefore the current data may indicate that haloperidol directly or indirectly alters the RNA editing machinery connected with ADAR 2 activity. ADAR 2 edits five members of the GluR gene family. In particular, the RNA edited Q/R-site of the α-amino-3-hydroxy-5-methylisoxazole-4-propionate receptor GluR2 subunit critically controls the calcium permeability of the associated ion channel (Lomeli et al., 1994). Many additional substrates for RNA editing are emerging (Levanon et al., 2004), and therefore a hitherto unidentified substrate could explain these findings.

Our data also have implications for neurogenetic studies of human disease. Postmortem findings have shown reduced GluR2 Q/R site editing and reduced 5-HT_{2C} RNA editing (at all sites) in schizophrenia (Akbarian et al., 1995; Sodhi et al., 2001). Although many of the patients in the latter study were not treated with haloperidol (Sodhi et al., 2001), it is possible that long-term antipsychotic treatment could have contributed to the RNA editing reduction in subjects with schizophrenia compared with the control subjects. However, the reduced RNA editing in subjects with schizophrenia was observed at all editing sites, and not just at the D site as in the haloperidol-treated rats. Additional studies of animals administered drug doses with greater equivalence and clinical relevance will clarify these findings, but the current data indicate that caution is required in the interpretation of RNA editing changes in tissue taken from patients exposed to long-term antipsychotic drug treatments. Furthermore we demonstrate that in vivo RNA editing can be modulated by an environmental, nongenetic factor (i.e., drugs) to interrupt the linear relationship between genotype and phenotype. This would not be detected by genomic DNA analysis and therefore could produce contradictory molecular genetic data in studies of complex hereditary diseases, such as schizophrenia.

Statistical Power and Data Clustering. Statistical power estimates of the current study deal with data clustering and support the need for a high-throughput method to measure RNA editing. It is estimated that to achieve 80% power to detect a small effect with medium clustering, the minimum number of subjects required would be 84 when 200 RNA molecules from each subject are tested. Although this is a large sample, using the new methodology, 16,000 clones could be processed using 178 $\times$ 96-well plates. The same task using DNA sequencing would be prohibitively lengthy and expensive. Circumventing this problem by pooling extracted RNA or cDNA from animals in a group to compare differences between groups ignores the environmental differences between animals that could create clustering in some variables measured. Clustering was observed in the current data when the level of B site editing was measured. Pooling of mRNA or cDNA would therefore preclude estimations of clustering for reliable statistical analyses.

Previous analyses of RNA editing data have proceeded as if the transcripts were statistically independent of the subject from which they were sampled (Sodhi et al., 2001). This is equivalent to assuming that the ICC = 0. Caution is warranted with this approach, because 1) the ICC confidence interval in previously published and current data often have an upper limit invalidating the assumption of independence, and 2) it can be argued that the appropriate null hypothesis for using a test that assumes independence should be that the ICC is less than a specific negligible value, which is analogous to a minimum-effect null hypothesis (Murphy and Myors, 2003). These statistical calculations depend on an accurate estimation of the ICC or clustering (Zou and Donner, 2004).

The presence of clustering will reduce the statistical power of a given sample size compared with the same sample size in which clustering is absent (i.e., the measures are independent). This reduction will depend on the level of the clustering present. With medium-sized data clustering (ICC = 0.1), the study had 99% power to detect large effects (effect size, w = 0.5) and greater than 95% power to detect medium effects (w = 0.3). However to detect small effects (w = 0.1), the presence of medium-sized clustering reduces the statistical power to approximately 25%. These statistical power estimations were made from the graphs in Fig. 3 and published tables of statistical power (Cohen, 1988). Figure 3 illustrates that if the ICC is large (>0.3), power rapidly becomes insensitive to additional clones per animal and that increased power is best achieved by the addition of animals or subjects (Hsieh, 1988). The use of models that allow for clustering should have at least 30 clusters (Kirkwood and Sterne, 2003b). In smaller samples, simpler analyses of summary statistics should be considered.

In conclusion, we have adapted a simple genotyping method to identify RNA edit variants and have addressed statistical issues associated with this research problem. There seems little doubt that RNA editing is widespread and is an important source of protein diversity (Levanon et al., 2004). The RNA-editing events detected in mammals have profound effects on protein function, and these are yet to be fully explored in the etiology of neurodegenerative and psychiatric disorders (Akbarian et al., 1995; Niswender et al., 2001; Sodhi et al., 2001; Gurevich et al., 2002; Dracheva et al., 2003). Furthermore, the current study provides evidence that risperidone and haloperidol alter 5-HT_{2C} RNA editing in different ways that may be related to their efficacy as antipsychotics. Therefore, we have developed an accurate, high-throughput tool for greater exploration of the pathological consequences of altered RNA editing. The importance of RNA editing is likely to increase as new substrates are identified, and it is possible that the novel RNA editing assay could be adapted in the future for the purpose of diagnosis or drug development.

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