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Quantitative analysis of 5HT_{2C} receptor RNA editing patterns in psychiatric disorders

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Abstract

Initially identified as an RNA modification in the anticodon loop of tRNAs from animal, plant and eubacterial origin, the deamination of adenosine-to-inosine by RNA editing has become increasingly recognized as an important RNA processing event to generate diversity in both the transcriptome and proteome and is essential for modulating the activity of numerous proteins critical for nervous system function. Here, we focus on the editing of transcripts encoding the 2C-subtype of serotonin receptor (5HT_{2C}) to generate multiple receptor isoforms that differ in G-protein coupling efficacy and constitutive activity. 5HT_{2C} receptors have been implicated in the regulation of anxiety, components of the stress response, and are thought to play a role in compulsive behavioral disorders, depression and drug addiction. A number of studies have been conducted to assess whether 5HT_{2C} editing is altered in individuals suffering from psychiatric disorders, yet the results from these studies have been inconsistent, and thus inconclusive. This review provides a discussion of the challenges involved with characterizing 5HT_{2C} editing patterns in human postmortem tissue samples and how differences in quantitative methodology have contributed to the observed inconsistencies between multiple laboratories. Additionally, we discuss new high-throughput sequencing tools, which provide an opportunity to overcome previous methodological challenges, and permit reliable systematic analyses of RNA editing in control and pathologic disease states.

Introduction

A major goal of current neurobiology research is to define and characterize the cellular and molecular pathophysiology underlying nervous system dysfunction including neurodegenerative disorders and psychiatric illness. Over the past two decades, a fundamental component of this effort has involved human postmortem brain studies where gene expression profiles of matched tissue samples from healthy individuals and patients diagnosed with specific nervous system disorders have been compared (Horvath et al., 2011; Iwamoto and Kato, 2006; Mehta et al., 2010; Sequeira and Turecki, 2006). While this traditional approach can be confounded by a number of variables such as postmortem

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interval, medication history, secondary effects of illness, cause of death and the small number of brain samples available for analysis (Bahn et al., 2001; Mirnics et al., 2004; Mirnics and Pevsner, 2004), technical artifacts of gene expression analysis may also contribute to inconsistencies between published datasets among multiple laboratories. The majority of transcriptome-wide gene expression studies have taken advantage of microarray strategies to simultaneously compare the relative expression of thousands of RNAs across sets of tissue samples. A limitation to this probe-based approach results from the inherent requirement to design probes based upon known (or predicted) sequences for genes of interest. The observation that a majority of human genes give rise to multiple mRNA isoforms by alternative splicing (Pan et al., 2008; Wang et al., 2008) or RNA editing (Gott and Emeson, 2000; Hogg et al., 2011; Zinshteyn and Nishikura, 2009) has further complicated these analyses as early microarrays typically contained probes consisting of full-length cDNAs or oligonucleotide probes located towards the 3' end of transcripts which were unable to distinguish alternatively spliced or closely-related mRNA species. More recent microarray platforms have been developed to distinguish between splice variants by using either a) tiling arrays, consisting of overlapping probes across a known genomic region (Kwan et al., 2008); b) exon arrays, consisting of probe sets corresponding to annotated and predicted exons (Clark et al., 2007; Gardina et al., 2006); c) splice-junction arrays, consisting of probes crossing splice junctions (Castle et al., 2003; Johnson et al., 2003); or d) exon-junction arrays, consisting of probes within exons as well as across exon junctions (Fagnani et al., 2007; Pan et al., 2004). Despite these advances in expression profiling for alternatively spliced variants, no probe-based strategies have been developed to quantify RNA editing events where modifications may result in as little as a single nucleotide alteration between RNA isoforms. More recently, non-probe based approaches such as serial analysis of gene expression (SAGE) (Scott and Chrast, 2001; Velculescu et al., 2000; Yamamoto et al., 2001) and massively parallel high-throughput sequencing (deep-sequencing) platforms (Marioni et al., 2008; Mortazavi et al., 2008; Wang et al., 2009) have allowed *de novo* analysis of transcript composition within RNA samples, providing a more unbiased and quantitative analysis of gene expression. These advances in sequencing technology have led to the development of whole-transcriptome profiling strategies, often referred to as RNA-Seq (Haas and Zody, 2010; Liu et al., 2011; Lovci et al., 2011; Mortazavi et al., 2008; Nagalakshmi et al., 2010; Wilhelm et al., 2010), where junctions between exons can be assayed without prior knowledge of the gene structure, subtle modifications resulting from RNA editing events can be readily detected and knowledge of polymorphisms can provide direct measurements of allele-specific expression. These approaches have led to an increased appreciation for the extensive sequence diversity generated by RNA processing events such as splicing and editing (Li et al., 2011) and provide powerful tools for more detailed analyses of changes in gene expression associated with human disease.

Post-transcriptional modification of transcripts by RNA editing

The conversion of adenosine-to-inosine (A-to-I) by RNA editing is a post-transcriptional processing event in which genomically-encoded sequences are altered in precursor and mature mRNAs to generate functional diversity in the proteome (Gott and Emeson, 2000). A-to-I editing is generally identified as an adenosine-to-guanosine (A-to-G) discrepancy during comparisons of genomic and cDNA sequences that result from the base-pairing of cytosine to inosine during reverse transcriptase-mediated first-strand cDNA synthesis. Because inosine is recognized as guanosine during translation (Basilio et al., 1962), adenosine deamination has been shown to "recode" amino acid sequences for key regulators of nervous system function including ligand- and voltage-gated ion channels, G-protein coupled receptors and components of the synaptic release machinery (Burns et al., 1997; Hoopengardner et al., 2003; Li et al., 2009; Schaub and Keller, 2002; Seeburg and Hartner,

2003; Zinshteyn and Nishikura, 2009). Indeed, A-to-I editing generally alters residues that are both highly conserved and critical for modulating the function of the encoded protein products (Jepson and Reenan, 2008; Rula and Emeson, 2007). Such alterations in the primary nucleotide sequence of mRNA transcripts can affect not only coding potential, but also can occur in untranslated regions and introns to alter the structure, stability, translation efficiency and splicing patterns of the modified transcripts, thereby affecting numerous aspects of RNA function in the cell (Gott and Emeson, 2000).

The first example of A-to-I editing in mammalian mRNAs was identified in transcripts encoding the GluR-2 subunit of the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subtype of ionotropic glutamate receptor in which a genomically-encoded glutamine codon (CAG) was altered to an arginine codon (CIG) (Melcher et al., 1995; Rueter et al., 1995; Sommer et al., 1991; Yang et al., 1995). This single amino acid alteration (Q/R site) regulates both the electrophysiologic and ion-permeation properties of heteromeric AMPA receptors. AMPA channels containing the edited GluR-2(R) subunit are impermeant to calcium ions, whereas those that lack or contain a non-edited GluR-2(Q) subunit, demonstrate a dramatic increase in their relative divalent cation permeability (Dingledine et al., 1992; Hollmann et al., 1991; Sommer et al., 1991; Verdoorn et al., 1991). Mice that are heterozygous for a mutant GluR-2 allele that cannot be edited appear healthy until postnatal day 14 (P14), when they begin to develop seizures that lead to death by P20. This phenotype results from dramatically increased AMPA receptor permeability to Ca^{2+} , concomitant with neuronal degeneration, and emphasizes the importance of RNA editing for nervous system function (Brusa et al., 1995).

Several lines of evidence have suggested that site-specific A-to-I editing is regulated spatiotemporally (Hackler et al., 2006; Jacobs et al., 2009; Niswender et al., 1998; Wahlstedt et al., 2009) and can be influenced by both genetic (Du et al., 2006) and environmental factors (Englander et al., 2005). The profound effect that A-to-I editing can have on protein function makes it essential to understand how the editing of specific RNA substrates is regulated in both normal and disease states. Efforts to gain a more complete picture of editing dynamics require methods to systematically and accurately determine the profile of edited RNA isoforms within experimental samples. This review provides a critical survey of approaches that have been used to compare editing profiles for transcripts encoding the 2C-subtype of serotonin receptor in postmortem brain tissue from healthy individuals and patients diagnosed with psychiatric disorders. Furthermore, it will discuss current and future approaches that will provide an opportunity to understand the biological significance of RNA editing in normal and pathological brain function.

Modulation of serotonin 2C receptor function by RNA editing

Serotonin (5-hydroxytryptamine; 5HT) is a monoaminergic neurotransmitter that modulates numerous sensory and motor processes as well as a wide variety of behaviors including sleep, appetite, pain perception, locomotion, thermoregulation, hallucinations, and sexual behavior (Werry et al., 2008). The multiple actions of 5HT are mediated by specific interaction with multiple receptor subtypes. Pharmacological, physiologic and molecular cloning studies have provided evidence for fifteen distinct 5HT receptor subtypes which have been subdivided into seven families (5HT₁–5HT₇) based on relative ligand binding affinities, genomic structure, amino acid sequence similarities and coupling to specific signal transduction pathways (Barnes and Sharp, 1999; Bockaert et al., 2006; Hoyer et al., 1994; Hoyer et al., 2002). The 5HT₂ family of receptors includes three receptor subtypes: 5HT_{2A}, 5HT_{2B} and 5HT_{2C}, which belong to the G-protein-coupled receptor (GPCR) superfamily. The G-protein:5HT_{2C} receptor interactions occur in highly conserved regions of the second and third intracellular loops to potentiate subsequent signal transduction

pathways via $G_{q/11}$, $G_{12/13}$ and G_{α_i} to modulate effector molecules such as phospholipases C, D and A_2 , as well as the extracellular signal-regulated kinases 1 and 2 (Berg et al., 1994; Berg et al., 1998; Werry et al., 2005; Werry et al., 2008). $5HT_{2C}$ mRNA expression has been shown to be widely distributed in neocortical areas, hippocampus, nucleus accumbens, amygdala, choroid plexus, dorsal striatum and substantia nigra (Pasqualetti et al., 1999; Pompeiano et al., 1994), suggestive of physiologic roles in reward behavior, locomotion, energy balance, and also when dysregulated, in the development of certain disease states such as obesity, epilepsy, anxiety, sleep disorders and motor dysfunction (Giorgetti and Tecott, 2004). Many of these anatomical predictions for $5HT_{2C}$ function have been supported by analyses of $5HT_{2C}$ -null mice that exhibit adult-onset obesity, seizures and enhanced cocaine-mediated locomotor activity and reward behavior (Abdallah et al., 2009; Brennan et al., 1997; Giorgetti and Tecott, 2004; Rocha et al., 2002; Tecott et al., 1995). Pharmacologic data have provided further support for these functional roles as selective $5HT_{2C}$ agonists promote appetite suppression (Halford et al., 2007; Somerville et al., 2007), facilitate anxiety-like behaviors (Harada et al., 2006; Kennett et al., 1997), reduce cocaine-mediated locomotion and drug self-administration (Fletcher et al., 2004; Harada et al., 2006) and have been implicated in the pharmacological actions of both hallucinogens and atypical antipsychotics (Giorgetti and Tecott, 2004; Marquis et al., 2007; Siuciak et al., 2007).

RNA transcripts encoding the $5HT_{2C}$ receptor undergo up to five A-to-I editing events that predict alterations in the identity of three amino acids within the second intracellular loop of the receptor to generate as many as twenty-four receptor isoforms from thirty-two edited mRNA species (Fig. 1) (Burns et al., 1997; Wang et al., 2000). Sequence analysis of cDNAs isolated from dissected rat, mouse and human brains predicted the region-specific expression of seven major $5HT_{2C}$ isoforms encoded by eleven distinct mRNA (Abbas et al., 2010; Burns et al., 1997; Morabito et al., 2010b; Niswender et al., 1998), suggesting that differentially edited $5HT_{2C}$ receptors may serve distinct biological functions in those regions in which they are expressed. Sequencing studies have further revealed that edited mRNAs encoding isoforms with valine, serine and valine (VSV) or valine, asparagine and valine (VNV) at amino acids 156, 158 and 160 are the most highly expressed in a majority of dissected brain regions isolated from human and rat/mouse brains, respectively (Burns et al., 1997; Fitzgerald et al., 1999), whereas the major $5HT_{2C}$ transcripts in the choroid plexus encode the less edited (INV) and non-edited (INI) receptor isoforms (Burns et al., 1997; Morabito et al., 2010b). Functional comparisons in heterologous expression systems, between the non-edited (INI) and the fully-edited (VGV) $5HT_{2C}$ isoforms have revealed a 40-fold decrease in serotonergic potency to stimulate phospho-inositide hydrolysis for the VGV isoform due to reduced $G_{q/11}$ -protein coupling efficiency and decreased coupling to other signaling pathways (Burns et al., 1997; Niswender et al., 1999). In addition, cells expressing more highly edited $5HT_{2C}$ receptors (e.g. VSV and VGV) demonstrate considerably reduced (or absent) constitutive activation in the absence of ligand compared to cells expressing the non-edited isoform (Niswender et al., 1999). This reduction in coupling efficiency and constitutive activity derives from a difference in the ability of edited $5HT_{2C}$ isoforms to spontaneously isomerize to the active R^* conformation, a form of the receptor that interacts efficiently with G-proteins in the absence of agonist (Burns et al., 1997; Niswender et al., 1999). Mutant mice solely expressing the fully-edited (VGV) isoform of the receptor pheno-copy numerous aspects of Prader-Willi Syndrome (PWS) (Kawahara et al., 2008; Morabito et al., 2010a), a maternally-imprinted human disorder resulting from a loss of paternal gene expression on chromosome 15q11-13 which is characterized by a complex phenotype including cognitive deficits, infantile hypotonia and failure-to-thrive, short stature, hypogonadism and hyperphagia which can lead to morbid obesity (Goldstone, 2004; Nicholls and Knepper, 2001). The dramatic phenotypic alterations observed in these mutant animals clearly demonstrate the importance of normal patterns of $5HT_{2C}$ RNA

editing *in vivo* and suggest a potential role for altered 5HT_{2C} function in the etiology of this imprinted disorder (Kawahara et al., 2008; Morabito et al., 2010a).

5HT_{2C} pre-mRNA editing in human psychiatric disease

Serotonin signaling has been implicated widely in the etiology of behavioral and psychiatric disorders and several 5HT receptors, including the 5HT_{2C} receptor, are thought to be important targets for pharmacologic intervention. In humans, antipsychotic drugs used in the treatment of schizophrenia interact with 5HT_{2C} receptors (Meltzer, 1999; Siuciak et al., 2007) and the 5HT_{2C} receptor has been hypothesized to play a role in psychiatric impairment associated with depression, anxiety and schizophrenia (Giorgetti and Tecott, 2004). For these reasons, several research groups have examined potential changes in 5HT_{2C} editing patterns to determine whether alterations in this RNA processing event play a role in the neuronal processes leading to psychiatric dysfunction. While multiple reports have provided evidence for changes in editing within human disease cohorts, and in rodents exposed to various pharmacologic and behavioral paradigms, such reports have been inconsistent and no reproducible alterations in editing have been effectively associated with any particular human disease cohort (Dracheva et al., 2008; Gurevich et al., 2002a; Gurevich et al., 2002b; Iwamoto and Kato, 2002; Iwamoto and Kato, 2003; Niswender et al., 2001; Sodhi et al., 2001). This is likely due to several factors including inconsistency in the precise brain regions analyzed, inherent diversity in human populations, and technical limitations that have made it difficult to efficiently and accurately determine the profile of edited 5HT_{2C} transcript isoforms in postmortem tissue samples.

The most common methodology to quantify site-specific editing has involved reverse-transcription polymerase chain reaction (RT-PCR) amplification of specific RNAs followed by a modified primer-extension analysis (Burns et al., 1997; Rueter et al., 1995); however, this analytical paradigm is less useful for analyses of editing patterns in transcripts with multiple editing sites, such as 5HT_{2C} mRNAs. The two more commonly used methods to quantify editing of 5HT_{2C} RNA isoforms involve either direct sequencing or pyrosequencing analysis of individual cDNA clones generated by RT-PCR and subcloned into plasmids (Iwamoto et al., 2005; Sodhi et al., 2005; Werry et al., 2008). While these methods yield unambiguous results, the accuracy of such methods is directly related to the number of clones sequenced, which can vary widely between studies and has been reported to be as few as 10 clones per sample (Sodhi et al., 2001). The limited number of clones analyzed can produce significant sampling errors that may either obscure or overestimate differences between experimental groups. Recent studies have analyzed 5HT_{2C} editing using 454 deep sequencing technology, increasing the number of 5HT_{2C} cDNAs sequenced to nearly 800, while simultaneously avoiding the laborious subcloning, bacterial transformation and preparation of individual cDNA clones (Wahlstedt et al., 2009).

Most studies attempting to determine the editing profile for 5HT_{2C} transcripts in postmortem brain samples from patients diagnosed with psychiatric disorders have focused primarily on RNA isolated from regions of the dorsolateral prefrontal cortex (DLPFC), specifically Brodmann areas 8, 9, 10, and 46. The first of these studies (Niswender et al., 2001) analyzed RNA in postmortem tissue (Brodmann areas 8 and 9) from individuals diagnosed with major depressive disorder, schizophrenia and healthy, age-matched controls. This study relied upon primer-extension-based approaches to quantify the extent of editing at the A, C, and D sites, yet revealed no significant differences in RNA editing among the three populations. However, subjects who had committed suicide (regardless of diagnosis) exhibited a statistically significant elevation of editing at the A-site. A contemporaneous examination of 5HT_{2C} editing patterns in DLPFC samples (Brodmann area 46) from schizophrenic and control subjects obtained from the Stanley Foundation Brain Collection

(Torrey et al., 2000) employed direct sequencing of individual cDNA isolates to quantify RNA editing patterns (Sodhi et al., 2001). In this approach, only 10 cDNA clones were sequenced from each individual to estimate changes among the thirty-two potential 5HT_{2C} isoforms that can be generated by editing. Results from this study reported a significant reduction in editing at all five sites in the DLPFC from individuals diagnosed with schizophrenia compared to controls and the authors suggested that observed differences from the results reported by Niswender (Niswender et al., 2001) could be explained as a result of different cortical regions being used for these analyses. A third research group (Gurevich et al., 2002b) used the sequencing of individual cDNA clones to compare 5HT_{2C} editing profiles in DLPFC (Brodmann area 10) of suicide victims with a history of major depression to that of non-depressed controls. These investigators used the same direct sequencing strategy employed by Sodhi and colleagues (Sodhi et al., 2001), but attempted to increase the accuracy of their estimates by analyzing 30–60 clones per tissue sample, rather than the 10 clones reported previously. These studies identified a significant increase in C-site editing and a decrease in D-site editing in depressed suicide victims compared to controls. Interestingly, they also quantified 5HT_{2C} editing patterns in the forebrain neocortex of mice treated for 28 days with oral fluoxetine and reported that drug treatment lead to changes in editing at the C- and D-sites that were opposite to those observed in depressed suicide victims. The authors suggested that this result supports a model where C- and D-site editing could be important for the etiology of depression and that compensatory alterations in editing efficiency at these sites may play a role in the therapeutic efficacy of the antidepressant, fluoxetine (Gurevich et al., 2002b). A fourth study examined RNA editing efficiencies of the A- and D-sites in 5HT_{2C} transcripts in prefrontal cortex samples of patients diagnosed with bipolar disorder, schizophrenia, and major depression using primer-extension combined with denaturing high performance liquid chromatography. Results from these analyses did not find significant alterations in RNA editing between patients and controls, although trends for increased RNA editing at the D-site in depressive patients (P=0.08) and the A-site in suicide victims (P=0.07) was noted. These results disagree with those described in the Gurevich study (Gurevich et al., 2002b), despite the fact that both studies quantified 5HT_{2C} editing in RNA samples isolated from Brodmann area 10. Finally, more recent studies (Dracheva et al., 2008) analyzed editing in DLPFC (Brodmann area not specified) by sequencing 45 clones per individual with cohorts of 82 normal controls and 22 suicide victims to report significant increases in the efficiency of editing at the A-, C-, and D-sites in suicide victims compared to controls (Dracheva et al., 2008). The discordant results between these studies raise significant concerns regarding the well-known limitations of postmortem brain analysis (Bahn et al., 2001; Mirnics et al., 2004; Mirnics and Pevsner, 2004) as well as the use of inadequate methodologies for effective quantification of 5HT_{2C} editing patterns.

New approaches for quantification of editing profiles

In 2006, Sodhi and colleagues described a pyrosequencing-based paradigm to quantify 5HT_{2C} editing patterns that circumvented some of the laborious steps previously employed for direct sequence analysis of individual cDNA clones (Sodhi et al., 2005). These studies also provided a careful analysis of the number of clones that need to be sequenced per RNA sample to detect 5HT_{2C} editing changes of different magnitudes, indicating that to detect “small” changes in editing between samples with adequate power (>80% confidence) several hundred clones would need to be analyzed from each sample. Recently, quantitative strategies have been developed to take advantage of deep-sequencing technologies that can provide simultaneous sequence identities for thousands to millions of PCR amplicons. These deep-sequencing approaches can be multiplexed for simultaneous analysis of editing from multiple RNA samples or from multiple RNA targets in several samples. Analyzing thousands of sequences from each sample provides sufficient statistical power to identify

small changes in editing with high confidence (Abbas et al., 2010; Morabito et al., 2010b) while reducing sample preparation, overall cost and time to facilitate analysis of large numbers of samples. This approach allows for a more detailed and accurate analysis of potential editing alterations in human disease cohorts and experimental animal models leading to new insights concerning the biological significance RNA editing.

Deep-sequencing analysis of 5HT_{2C} editing patterns takes advantage of the Illumina Genome Analyzer platform to directly sequence RT-PCR amplicons derived from 5HT_{2C} transcripts. This is achieved by designing sense and antisense oligonucleotide primers containing a region of complementarity to the target RNA and 5'-extensions that correspond to the adapter sequences required for bridge amplification and direct paired-end sequencing on the Illumina platform (Fig. 2) (Bentley et al., 2008). This technique can be multiplexed by including unique "barcode" sequences into the antisense PCR primer which provides maintenance of sample identity. This multiplexing strategy allows for the combination of many samples into one sequencing run, taking full advantage of the vast number (>10 million) of sequencing reads which are simultaneously generated on the Illumina platform. This large number of sequencing reads provides investigators with the opportunity to make more accurate estimates of the overall 5HT_{2C} isoform profile in a specific RNA sample and allows for enhanced detection of rare sequence variants (Abbas et al., 2010; Morabito et al., 2010b). Comparisons of 5HT_{2C} editing profiles in RNA samples isolated from whole C57BL/6J mouse brain using pyrosequencing and deep-sequencing approaches revealed significantly decreased experimental variability using the high-throughput approach (Morabito et al., 2010b). Quantification of 5HT_{2C} editing patterns using 100 cDNA clones from each of five brain samples by pyrosequencing suggested significant intra-animal variability, whereas the same five RNA samples analyzed using the Illumina-based strategy (>100,000 sequences per sample) decreased inter-animal variability by more than 10-fold (<3 % of mean). These results demonstrate that there is very little inter-animal variability in whole brain 5HT_{2C} isoforms when controlled for age, gender, and background strain in matched mouse cohorts (Morabito et al., 2010b) and suggests that similar analyses of human brain samples may be systematically applied, if appropriately controlled for other confounding variables. Furthermore, the overestimation of inter-sample differences observed when analyzing 100 clones illustrates that a degree of caution must be taken when interpreting estimates of 5HT_{2C} editing patterns that are based on tens or even hundreds of clones.

Conclusion

Previous studies characterizing 5HT_{2C} editing in the human brain have relied upon crude dissections of human cortex corresponding to either specific Brodmann areas or even less well defined samples of cortical tissue. Given the discordant results obtained by many laboratories investigating potential alterations in 5HT_{2C} editing, it is increasingly unclear whether dynamic changes in editing are associated with 5HT_{2C} genotype, expression level, medication history, or psychiatric diagnosis. While analyses of human postmortem brain samples have a number of limitations, the development of accurate and sensitive methodologies for quantifying 5HT_{2C} editing patterns represents a critical first-step to minimize technical artifacts associated with inadequate sample size or extensive variability within and between isolated RNA samples. The advent of high-throughput sequencing approaches and high-precision tissue sampling techniques like laser-capture microdissection, offers opportunities to obtain precise site-specific editing profiles for individual neurons allowing comparisons of normal and pathological states.

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Figure 1. Structures of the human 5HT_{2C} gene, mRNA, receptor protein and RNA editing events
A) A schematic diagram of the human 5HT_{2C} receptor gene (*Htr2C*) is mapped onto the mature mRNA transcript; the positions of the mRNA encoding the transmembrane domains (TM), the untranslated regions (UTR) and the sizes of exon and introns (bp, base pairs) are indicated. **B)** Schematic representation of the predicted topology and primary amino acid sequence for the human 5HT_{2C} receptor is presented along with the positions of amino acid alterations within the second intracellular loop resulting from RNA editing events (*colored circles*). **C)** Nucleotide and predicted amino acid sequence alignments between 5HT_{2C} genomic, mRNA and cDNA sequences; the positions of the five editing sites (A-E) are indicated and nucleotide discrepancies and predicted alterations in amino acid sequence are shown with colors corresponding to each codon in which they reside.

Figure 2. Summary of the high-throughput sequencing strategy for analysis of 5HT_{2C} editing profiles

The structure of 5HT_{2C} pre-mRNA in the exon 5–6 region is presented (*top*). The location of RT-PCR primers are indicated (black arrows). Adapter sequences for paired-end sequencing on the Illumina Genome Analyzer (dark blue) were incorporated into each primer and one of eight distinct barcode sequences (yellow) was incorporated into the antisense primer. The sequence of the non-edited RNA, beginning immediately after the primer sequence, is specified below the pre-mRNA RT-PCR diagram. The positions of the five edited adenosines are indicated in bold (sites A, B, E, C and D) and the amino acids encoded by the non-edited (upper) and fully edited transcripts (lower) are indicated below their respective codons. The position of the 36nt sequence obtained from read 1 is indicated between the dashed lines on both the RT-PCR and pre-mRNA diagrams. Two sequencing primers (read 1 and read 2, red arrows) were used for paired-end sequence analysis of RT-PCR amplicons generated from 5HT_{2C} mRNA (*bottom*). Each of eight unique barcode sequences incorporated into the RT-PCR amplicon for sample identification is provided in the yellow inset.