Editing of Serotonin 2C Receptor mRNA in the Prefrontal Cortex Characterizes High Novelty Locomotor Response Behavioral Trait

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Abstract

Serotonin 2C receptor ($5-HT_{2C}R$) exerts a major inhibitory influence on dopamine (DA) neurotransmission within the mesocorticolimbic DA pathway that is implicated in drug reward and goal-directed behaviors. $5-HT_{2C}R$ pre-mRNA undergoes adenosine-to-inosine editing generating numerous receptor isoforms in brain. Because editing influences $5-HT_{2C}R$ efficacy, individual differences in editing might influence dopaminergic function and, thereby, contribute to inter-individual vulnerability to drug addiction.

Liability to drug-related behaviors in rats can be predicted by the level of motor activity in response to a novel environment. Rats with a high locomotor response (high responders; HRs) exhibit enhanced acquisition and maintenance of drug self-administration compared to rats with a low response (low responders; LRs). Here we examined $5-HT_{2C}R$ mRNA editing and expression in HR and LR phenotypes in order to investigate the relationship between $5-HT_{2C}R$ function and behavioral traits relevant to drug addiction vulnerability. Three regions of the mesocorticolimbic circuitry (ventral tegmental area (VTA), nucleus accumbens (NuAc) shell, and medial prefrontal cortex (PFC)) were examined.

$5-HT_{2C}R$ mRNA expression and editing was significantly higher in NuAc shell compared to both PFC and VTA, implying significant differences in function (including constitutive activity) among $5-HT_{2C}R$ neuronal populations within the circuitry. The regional differences in editing could, at least in part, arise from the variations in expression levels of the editing enzyme, ADAR2, and/or from the variations in the ADAR2/ADAR1 ratio observed in the study. No differences in the $5-HT_{2C}R$ expression were detected between the behavioral phenotypes. However, editing was higher in the PFC of HRs *vs.* LRs, implicating this region in the pathophysiology of drug abuse liability.

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Keywords

serotonin 2C receptor; mRNA editing; drug addiction; rat; high and low responders; prefrontal cortex

INTRODUCTION

Humans and animals show wide inter-individual differences in the susceptibility to drug addiction (de Wit et al., 1986; Piazza et al., 1998) with notably inherent dissimilarities in the reinforcing effects of addictive drugs (Volkow et al., 2002). The mesoaccumbal and mesocortical dopamine (DA) pathways, which originate in the ventral tegmental area (VTA) and terminate in the nucleus accumbens (NuAc) and prefrontal cortex (PFC), respectively, are most widely implicated in drug reward and goal-directed behaviors (Everitt and Robbins, 2005). Serotonin (5-HT) exerts a major modulatory influence on DA neurotransmission within these pathways via multiple 5-HT receptor subtypes (Roth, 1994). Among them, the 2C receptor subtype ($5-HT_{2C}R$) is of particular importance (Giorgetti and Tecott, 2004). This receptor is localized in brain regions containing both DA cell bodies and their terminations, where it modulates DA-dependent behavioral and neurochemical effects of a variety of addictive drugs (Berg et al., 2008). Numerous studies demonstrated that the systemic administration of $5-HT_{2C}R$ agonists inhibits the rewarding and psychomotor-stimulant effects of cocaine, amphetamine, nicotine, and ethanol, while $5-HT_{2C}R$ antagonists potentiate these effects (Grottick et al., 2001; Fletcher et al., 2002; Tomkins et al., 2002). In line with these behavioral data, the $5-HT_{2C}R$ exerts an inhibitory influence on both tonic and phasic DA neuron firing. Specifically, the basal and drug-stimulated firing rate of DA neurons in the VTA, as well as DA release at their terminals, are increased by the administration of the $5-HT_{2C}R$ antagonists and decreased by the administration of the receptor agonists (Gobert et al., 2000; Di Giovanni et al., 2002; Di Matteo et al., 2004; Navailles et al., 2004).

The $5-HT_{2C}R$ is the only known G-protein coupled receptor whose pre-mRNA undergoes adenosine to inosine (A-to-I) editing (Burns et al., 1997; Werry et al., 2008). This editing is catalyzed by enzymes termed *adenosine deaminases* that act on *RNA* (ADAR1 and ADAR2) (Bass, 2002; Schmauss and Howe, 2002; Valente and Nishikura, 2005). The ADARs can modify one or more of five closely spaced adenosine residues (named A, B, E, C, and D editing sites) on the receptor mRNA. These five adenosine residues are spread among three triplet codons within a sequence that encodes the putative second intracellular domain of the $5-HT_{2C}R$ protein -- a region thought to be important for G-protein coupling (Moro et al., 1993). Because inosine is read as guanosine by the translational machinery, $5-HT_{2C}R$ mRNA editing can alter the coding potential of the three codons generating up to 32 different mRNA variants and, consequently, up to 24 different protein isoforms. Previous *in vitro* studies indicated that RNA editing has a dramatic impact on the functional characteristics of $5-HT_{2C}R$. For example, compared with the non-edited receptor (INI), the two fully-edited receptor isoforms (VSV and VGV) exhibit decreased potency, ligand-independent (constitutive) activity, agonist affinity, and internalization (Niswender et al., 1999; Herrick-Davis et al., 1999; Fitzgerald et al., 1999; Wang et al., 2000; Berg et al.,

2001; Quirk et al., 2001; Marion et al., 2004). A mixture of differently edited and non-edited 5-HT$_{2C}$R mRNAs has been detected in both human and animal brains (Burns et al., 1997; Niswender et al., 2001). Individual differences in editing might, therefore, lead to differences in 5-HT$_{2C}$R function that could, in turn, influence dopaminergic function and contribute to inter-individual vulnerability to drug addiction.

Variations in the liability to drug abuse-related behavior have been demonstrated to occur naturally in outbred strains of rats. Moreover, liability to drug intake behavior is highly correlated with an animal's level of motor activity in stressful situations, such that drug self-administration as well as other behavioral and neurochemical responses to a psychostimulant can be predicted by the level of motor activity in response to a novel environment. Rats with a high locomotor response (high responders; HRs) have been well documented to exhibit enhanced acquisition and maintenance of drug self-administration and other drug-related behaviors compared with rats with a low response to the novel context (low responders; LRs) (Piazza et al., 1989; Pierre and Vezina, 1997). In the present study, we examined 5-HT$_{2C}$R editing and related mRNA transcripts in HR and LR animals in order to draw inferences regarding the relationship between 5-HT$_{2C}$R editing and behavioral traits relevant to drug addiction. Three regions of the mesocorticolimbic DA pathway (VTA, NuAc shell and medial PFC) were examined. In addition to region-specific variations in 5-HT$_{2C}$R mRNA editing common to both phenotypes, increased editing was seen in the PFC of HRs compared to LRs.

METHODS

Animal Housing and behavioral experiments

Six-week old male Sprague Dawley rats (N=104; Taconic) were processed in two sets of 52 animals each; the two series were separated by approximately 4 weeks. The animals were housed individually, maintained on 12 hr dark/ light cycle with food and water available *ad libitum*, and were allowed 2 weeks to acclimate to the animal room before the behavioral assessment. To determine the behavioral phenotype, rats were screened for locomotor response to novelty in open field apparatuses (VersaMax, Accuscan Instruments) for a 1 hr period as previously described (Marinelli and White, 2000). Each set of 52 animals was screened over 5 consecutive days at the same time of the day (between 11am and 1pm). All experiments were done in compliance with the Institutional Animal Care and Use Committee.

Tissue Sampling with Laser Capture Microdissection (LCM)

Tissue preparation—One week after the end of behavioral testing, animals designated as HRs or LRs were sacrificed by decapitation. Brains were removed, immediately frozen on dry ice, and subsequently sectioned serially in the coronal plane at a thickness of 20 μm in a cryostat (Leica CM 1900; Leica Microsystems Inc, Bannockburn, IL). For anatomical localization, the first two of each series of 10 sections were mounted onto regular glass slides, stained with thionin and cover-slipped to serve as reference sections. The next set of 8 sections were mounted onto penfoil polymer (PEN) microdissection slides and stored at

-80°C until use. Every effort was made to maintain a near surgical aseptic setting when handling brains, slides, and tissue sections to protect against RNA degradation by RNAses.

Anatomical parcellation—Three brain regions (PFC, VTA, NuAc) were microdissected from each experimental animal (Fig. 1). The PFC dissections were limited to laminae II-VI of the cortex on the medial bank of the frontal lobe at the level of the tenia tecta and contained primarily cingulate area 3 and the infralimbic cortex (approximately Bregma 2.2-3.2 mm) (Fig. 1a). NuAc dissections were taken from its shell region at the level of the genu of the corpus callosum (approximately Bregma 0.7-1.6 mm) (Fig. 1b). The VTA dissection was bounded by fasciculus retroflexus medially, the mammillary peduncle ventrally, the substantia nigra pars compacta and medial terminal nucleus of the accessory optic tract laterally, and the medial lemniscus dorsolaterally (approximately Bregma -5.2 - -5.3 mm) (Fig. 1c). While this dissection does not contain the entire VTA, it is highly reproducible and includes the region previously shown to express both tyrosine hydroxylase and 5-HT$_{2C}$R mRNAs (Eberle-Wang et al., 1997). Care was taken to exclude any portion of the substantia nigra from the dissections.

Laser capture microdissection (LCM)—On the day of microdissection, PEN slides at the anatomical level desired for dissection were identified by reference to nearly adjacent thionin-stained reference sections. The identified PEN slides were removed from the freezer, immediately fixed in 100% ethanol for 1 min and then rehydrated in a graded series of ethanol/water (95%, 75%, 50%; each one minute). Sections were then stained for ~ 20 sec in 1% Thionin in 0.05M NaCH$_3$COO buffer, pH 4.5, dehydrated (75%, 95%, 100% ethanols, 1 min each), dried for 1 min and immediately used for microdissection. All solutions were prepared with diethylpyrocarbonate (DEPC)-treated water. The individual regions were dissected by LCM using Leica AS LMD (Leica Microsystems Inc., Bannockburn, IL). The amount of dissected tissue varied for different regions due to the differences between their sizes. All delineations and dissections were performed blindly relative to the behavioral phenotypes of the animals.

RNA extraction and cDNA synthesis

Total RNA was extracted and treated with DNAse using the PicoPure RNA isolation Kit (Molecular Devices, Sunnyvale, CA). RNA quality and concentration were evaluated using BioAnalyzer (Agilent Technologies, Santa Clara, CA). All but two RNA preparations (one from the VTA and one from the NuAc) yielded high quality RNAs [MEAN±SD of the RNA integrity numbers (RIN) were 7.52±0.62], and only those high quality preparations were employed in the studies. cDNA was synthesized by reverse transcriptase (RT) reaction using iScript kit (BioRad, Hercules, CA). Since RNA concentrations varied between the different regions, different amounts of isolated RNA were used for RT reactions in each region: 400 ng (PFC), 100 ng (VTA), and 250 ng (NuAc). For a given region, equal amounts of RNA were used for each animal.

RNA editing analysis

Amplification of the 5-HT$_{2C}$R region of editing—To minimize the influence of erroneous amplification in a single PCR that could alter the proportions of differently edited

mRNA variants, three independent PCRs were performed for each animal and each region using Forward - ATCATGGCAGTAAGCATGG / Reverse - ATCTTCATGATGGCCTTAGTCCG primer-pair and a touch-down cycling program as described (Dracheva et al., 2003). Because the reverse primer was designed within the area that is deleted in the truncated 5-HT$_{2C}$R mRNA splice variant (5-HT$_{2C}$Rsp1; see below), only the full-length 5-HT$_{2C}$R (5-HT$_{2C}$Rsp2) cDNA was amplified in this PCR (Canton et al., 1996; Dracheva et al., 2003; Dracheva et al., 2008b).

Cloning and Sequencing—The PCR products of the three independent reactions generated for each animal and each brain region were combined and resolved by agarose gel electrophoresis. A single DNA product of ~ 300 bp was detected for each combined reaction. The amplicon of this size (302 bp) was expected from the full length 5-HT$_{2C}$R cDNA. The DNA product was gel-purified using a MinElute Gel Extraction Kit (Qiagen, Valencia, CA), and sub-cloned into a pCR4-TOPO vector (Invitrogen, Carlsbad, CA). The recombinant plasmids were transformed into bacteria. From each bacterial transformation, which resulted in at least 300 clones expressing the recombinant plasmid DNA, 48 were picked randomly. Plasmid DNA was isolated, and the inserts of these plasmids were sequenced. The plasmid DNA insert from a single bacterial colony represented the edited region of an individual 5-HT$_{2C}$R mRNA transcript.

Real time quantitative PCR (qPCR)

mRNA expression was measured by qPCR using an ABI Prism 7900 Sequence Detector and gene-specific TaqMan FAM/MGB assays (Applied Biosystems, Foster City, CA) as described (Dracheva et al., 2008a). Pre-developed TaqMan FAM/MGB assays (Applied Biosystems) were employed for the endogenous control genes (beta-2 microglobulin (B2M), Rn00560865_m1; peptidylprolyl isomerase A (PPIA), Rn00690933_m1; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Rn99999916_s1; beta-actin (BACT), Rn00667869_m1) amplification. Similarly, a pre-developed assay was used for ADAR1 (ADAR) (Rn00508006_m1) amplification, since only one splice variant is reported for this gene in rats [National Center for Biological Information (NCBI) Accession Number, NM_031006].

Four different splice variants of ADAR2 (ADARB1) are reported by the NCBI (see Results). Because two ADAR2 alternative splice sites are situated 1400 bp apart from each other, it was not feasible to design qPCR assays that would distinguish between all four individual transcript variants. Therefore, two assays were employed, which distinguished between the transcripts that encode the functional protein (denoted as ADAR2f) and the non-functional (truncated) protein (denoted as ADAR2nonf). A pre-developed TaqMan assay (Rn00681060_m1) was used to detect ADAR2f transcript variants. This qPCR assay amplified only transcript variants 1 and 2 (NM_012894; NM_001111055). A custom TaqMan assay was designed to detect ADAR2nonf variants. In this assay, the forward PCR primer was situated in the region of extra 47 bp that is added to the ADAR2-coding region as a result of auto-editing (see Results); therefore, only transcript variants 3 and 4 (NM_001111056, NM_001111057) were amplified (ADAR2nonf assay: F-

CTTTCTGGGCACCACAGGTT; R- GTTGTCCAGATTGCGGTTTTC; Probe- FAM-AGCAGCACTGATGTTAA -MGB).

Two different custom TaqMan assays were designed to distinguish between two 5-HT$_{2C}$R splice variants (5-HT$_{2C}$Rsp1 and 5-HT$_{2C}$Rsp2) that had been detected in the brain (see Results) (Dracheva et al., 2003; Flomen et al., 2004). In the 5-HT$_{2C}$Rsp2 assay, the probe and primers were situated inside the 95-bp region that is present in 5-HT$_{2C}$Rsp2, but is deleted in 5-HT$_{2C}$Rsp1; therefore, only 5-HT$_{2C}$Rsp 2 was amplified (5-HT$_{2C}$Rsp2 assay: F-TGAGCATAGCCGGTTCAATTC; R- GCCCAAACGATGGCAATCT; Probe-6FAM-CGGACTAAGGCCATCAT -MGB). In the 5-HT$_{2C}$Rsp1 assay, the forward PCR primer was designed to span the *sp1* site; therefore, only 5-HT$_{2C}$Rsp1 was amplified (5-HT$_{2C}$Rsp1 assay: FCGCTGGACCGGAGTTTCA; R- ATTCACGAACACTTTGCTTTCG; Probe-FAM-CCTATCCCTGTGATTGGA -MGB).

To account for the differences in the input material among the samples, the expression levels of 5-HT$_{2C}$R and ADAR2 were normalized to the expression levels of the four above-mentioned endogenous controls (B2M, PPIA, GAPDH, and BACT) (Vandesompele et al., 2002). First, the relative expression levels of all transcripts were determined using Relative Standard Curve Method (RSCM; see Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR, Applied Biosystems), which provides accurate quantitative results by accounting for differences in the efficiencies between the target and the endogenous control(s) amplifications (Peirson et al., 2003). The standard curves were generated by the association between threshold cycle (Ct) values and different quantities of a "calibrator" cDNA, which was prepared by mixing small quantities of all experimental samples. Using the linear equations of the standard curves, the relative amounts of the target transcripts (ADAR1, ADAR2f, ADAR2nonf, 5-HT$_{2C}$Rsp1, 5-HT$_{2C}$Rsp2) and the endogenous control transcripts were calculated in each sample. Then, the relative expression level of each target transcript was computed as the ratio between the amount of the target transcript and the geometric mean of the amounts of the four endogenous controls. The ADAR2f/ADAR1 ratio was calculated as the proportion between the relative expressions of ADAR2f and ADAR1.

Statistical analysis

Editing data—The editing parameters obtained in the study were: editing efficiencies at each of the five sites, number of each detected 5-HT$_{2C}$R mRNA variant, and number of each predicted 5-HT$_{2C}$R protein isoform. The data were analyzed using mixed-effects binomial regression (Hedeker and Gibbons, 1996) separately for each region as a function of phenotype (HRs or LRs). Editing efficiency was previously observed to exhibit a strong interdependence among all five sites (Carmel, Koonin and Dracheva, unpublished observation). Editing efficiencies at A, B, C, and D editing sites were, therefore, handled as repeated measures using a random effect. Due to the low editing efficiency (less than 5% in the PFC and VTA), the E site was not included in the analysis. The differences between regions were considered with HRs and LRs combined. The differences in editing efficiencies were confirmed using Tukey's Studentized Range Test.

Gene expression data—The relative mRNA expression of ADAR1, ADAR2f, ADAR2nonf, 5-HT$_{2C}$Rsp1, and 5-HT$_{2C}$Rsp2 and the ADAR2f/ADAR1 ratio were compared among the 3 brain regions for HRs and LRs by repeated measures analysis of variance (ANOVA) for the 35 subjects with complete data (17 HRs and 18 LRs). The Huynh-Feldt adjustment of degrees of freedom was used to correct for violation of sphericity assumptions. In addition, the HRs and LRs were compared separately within each brain region by one way ANOVA, and each pair of brain regions was compared by matched sample t-tests, including both HRs and LRs.

Correlation analysis—Correlations between the expression levels of ADARs and the editing efficiencies at all five editing sites, as well as correlations among ADARs' expression levels, were examined within each region and across all regions. Since a total of 28 correlations were examined, a criterion for significance was set at $p<0.05/28=0.0012$.

RESULTS

Evaluation of Behavioral Phenotypes

The weight of rats varied between 250 and 320 gm at the time of the behavioral testing and there were significant differences in weights between the 2 sets of animals (Mean ± SEM; set 1, 272±1.9 g; set 2, 284±2.7 g; $p<0.001$). Therefore, although no correlation was observed between the individual locomotor score and the weight of the animals (set1: $r=-0.088$, $p=0.57$; set2: $r=0.190$, $p=0.19$), weight was used as a covariate in the analysis of the locomotor scores. Analysis of covariance showed no differences between locomotor activity scores recorded for each daily group (10 groups, 10/12 animals per group; $F(1,9)=0.812$, $p=0.62$). Still, because two sets of rats were processed separately and analyzed at different times (4 weeks apart), the individual locomotor score for each rat was normalized by dividing it by the average of the activity counts for that set of animals. These adjusted scores were used for statistical analyses.

As in other studies (Marinelli, 2005), the normalized locomotor activity scores were approximately normally distributed (Kolmogorov-Smirnov $z=0.78$, $p=0.58$). High and low responder phenotypes were defined by the upper and lower ends of this distribution (approximately 1 SD above and 1 SD below the mean, respectively): From 104 animals assessed, 19 with the highest scores were designated as HRs and 19 with the lowest scores as LRs.

Analysis of RNA editing

Overall analysis—Nucleotide sequence analysis was obtained for a total of 5136 5-HT$_{2C}$R cDNA clones that were generated from the RNA specimens extracted from 3 different brain regions (PFC, NuAc shell, and VTA) of 38 rats (19 LRs and 19 HRs). Due to low concentration, 2 RNA specimens (1 from the VTA and 1 from the NuAc) were not analyzed. On average, 45.7 clones (range, 43-48) were analyzed for each animal in each region, which provided for a reliable analysis of editing as was determined in (Sodhi et al., 2005). The value of each RNA editing parameter (editing efficiency at each site, observed mRNA variants, and predicted protein isoforms) was calculated as the proportion of the

number of clones analyzed per a specimen. The mean values of these proportions (termed "frequencies" for the RNA variants and protein isoforms, and "efficiencies" for the levels of editing at each editing site) within each of the study groups (the entire group, LRs, and HRs) and within each brain region (PFC, NuAc shell, and VTA) are shown in Tables 1-3.

Twenty seven of the 32 possible 5-HT$_{2C}$R RNA variants were detected in the entire group among different regions *(BC, BE, BEC, BED,* and *BECD* were not detected). Only four of the detected variants, which contained editing combinations *ABD, ABCD, AB,* and *ABC,* were observed at ~5% or greater frequency in at least one of the study groups (Table 2). Taken together these four variants constituted the majority of the 5-HT$_{2C}$R transcripts (84.4% in the PFC, 85.4% in the VTA, and 83.9% in the NuAc shell). Of the 24 predicted 5-HT$_{2C}$R protein isoforms, RNA was detected for 19 (MDI, MDV, MGI, MSI, and MGV were not detected); only four of the predicted isoforms (VNV, VNI, VSV, and VSI) had a frequency of 5% or greater (Table 3). Taken together these four predicted isoforms represented approximately 90% of the 5-HT$_{2C}$R protein (90.8% in the PFC, 93.4% in the VTA, and 89.1% in the NuAc shell).

Comparison between HRs and LRs—The editing efficiency was significantly higher in HRs compared to LRs in the PFC (z=2.44, p=0.0145); no differences were detected in the VTA and NuAc shell (Table 1). Similarly, the frequency of *ABCD* was significantly higher for HRs only in the PFC (z=2.10, p=0.0352; Fig. 2A, Table 2). Consequently, the frequency of VSV (that is encoded by *ABCD*) was higher for HRs compared to LRs in the PFC (z=2.15, p=0.0315; Fig. 2B, Table 3). No differences between HRs and LRs were detected in the frequencies of *ABD, AB, ABC,* VNV, VNI, and VSI in any region (see Fig. 2A and 2B for *ABD* and VNV, respectively).

Comparison between the regions—The editing efficiency at the A, B, C, D sites (analyzed as repeated measures) was significantly higher in the NuAc shell compared to the PFC and VTA (z values >6.14, p values <0.0001; Fig. 3A). Tukey's Studentized Range Test showed that for site C, there was a significant increase of editing efficiency in the NuAc compared to the VTA (95% CI: 0.0056, 0.0916). For site D, there was a significant increase of editing in the NuAc compared to the VTA (95% CI: 0.1272, 0.2097), in the NuAc compared to the PFC (95% CI: 0.1755, 0.2575), and in the VTA compared to the PFC (95% CI: 0.0070, 0.0890).

The frequency of *ABD* was significantly lower in the PFC compared to the NuAc shell (z=3.28, p=0.001; Fig.3B). The frequency of *ABCD* was significantly higher in the NuAc shell compared to the PFC and VTA (z values >7.17, p values <0.0001; Fig. 3B). The frequencies of *AB* and *ABC* were significantly lower in the NuAc shell compared to the PFC and VTA (z values >6.89, p values <0.0001; Fig. 3B). The frequency of VNV was significantly lower in the PFC compared to the NuAc shell (z=4.21, p<0.0001) and VTA (z=2.49, p=0.013). The frequency of VSV was significantly higher in the NuAc shell compared to the PFC and VTA (z values >7.16, p values <0.0001; Fig. 3C). The frequencies of VSI and VNI were significantly lower in the NuAc shell compared to the PFC and VTA (z values >7.23, p values <0.0001; Fig. 3C).

Analysis of the 5-HT$_{2C}$R, ADAR1, and ADAR2 mRNA expression

In addition to a constitutive 5' splice site, *sp2,* 5-HT$_{2C}$R pre-mRNA undergoes alternative splicing between exons 5 and 6 with two alternative splice sites described -- *sp1* and *sp3* (Canton et al., 1996; Flomen et al., 2004). While the mRNA variant generated from splicing at *sp2* is translated into a functional receptor, both of the resulting alternative splice variants are translated into truncated proteins that are unlikely to retain adequate receptor function. The splicing at the *sp3* site was found to be extremely rare both in cell culture and in the brain (Dracheva et al., 2003; Flomen et al., 2004). Two custom TaqMan assays (see Methods) were employed in this study to detect each of the two 5-HT$_{2C}$R splice variants expressed in the brain -- 5-HT$_{2C}$Rsp1 and 5-HT$_{2C}$Rsp2 - that result from splicing at *sp1* and *sp2*, respectively.

Four different splice variants of ADAR2 (ADARB1), which originate from constitutive and 2 alternative splicing events, are reported by the NCBI. The mammalian ADAR2 pre-mRNA transcript is edited by its own protein to produce an alternative intronal 3'-splice site. This splice site adds an extra 47 nucleotides to the ADAR2 mRNA coding region creating a frame shift that results in a non-functional truncated protein (Rueter et al., 1999). In addition, ADAR2 pre-mRNA carries an alternative in-frame donor splice site in one of the internal coding exons, resulting in a shorter transcript that is missing a 10 aa segment. Transcript variant 1 (NM_012894) is the longest variant that does not undergo any alternative splicing and encodes a functional protein; transcript variant 2 (NM_001111055) uses an alternative in-frame donor splice site in the coding exon and encodes a functional protein that is 10 aa shorter than the first isoform; transcript variant 3 (NM_001111056) uses an alternative intronal splice site and encodes non-functional truncated protein; transcript variant 4 (NM_001111057) uses both alternative splice sites and also produces non-functional truncated protein product. Two assays were employed in this study to distinguish between the transcripts that encode functional (ADAR2f) and truncated non-functional (ADAR2nonf) proteins (see Methods). The ratio between ADAR2f and ADAR1 expression levels was also analyzed.

The repeated measures analysis showed very significant differences among regions for all transcripts and for the ADAR2f/ADAR1 ratio (5-HT$_{2C}$Rsp1, F=1115.17; df=1.47, 46.93; p<0.0001; 5-HT$_{2C}$Rsp2, F=1086.12; df=1.69, 53.92; p<0.0001; ADAR1, F=83.23; df=1.85, 59.14; p<0.0001; ADAR2f, F=239.89; df=1.48, 47.27; p<0.0001; ADAR2nonf, F=298.94; df=1.29, 41.29; p<0.0001; ADAR2f/ADAR1 ratio, F=27.86; df=1.79, 57.23; p<0.0001), but the differences between HRs and LRs and phenotype by region interaction were not significant. Similarly, the differences between HRs and LRs were not significant in any region, but the differences between pairs of regions (PFC *vs.* NuAc shell, PFC *vs.* VTA, and VTA *vs.* NuAc shell) were highly significant for all transcripts: (5-HT$_{2C}$Rsp1, all t values 16.03, all p values <0.0001; 5-HT$_{2C}$Rsp2, all t values 11.79, all p values <0.0001; ADAR1, all t values 6.11, all p values <0.0001; ADAR2f, all t values 9.96, all p values <0.0001; ADAR2nonf, all t values 13.70, all p values <0.0001 Figs. 4 and 5). The ADAR2f/ADAR1 ratios were significantly different between the NuAc shell and the two other regions (all t values 5.83, all p values <0.0001), but did not differ between the PFC and VTA (Fig. 5).

Correlations between ADARs' mRNA expression and editing efficiencies

There were no correlations between ADARs' expression and editing efficiencies when the correlation analysis was performed in each individual region. However, significant correlations were observed between the ADARs expression levels and the editing efficiency at site D (all r values > 0.540, all p values <0.0001) when the analysis was performed across all three regions combined (Table 4). In addition, strong correlations among all ADAR transcripts were detected when the analysis was performed across the regions (all r values > 0.823, all p values <0.0001).

DISCUSSION

This study provides the first extensive evaluation of 5-HT$_{2C}$R mRNA editing in relation to addiction susceptibility. Most investigations to date had only examined 5-HT$_{2C}$R mRNA editing in total brain or in one brain region. Examining three brain regions provided insight into regional variations in 5-HT$_{2C}$R mRNA editing. Significant between region differences in 5-HT$_{2C}$R editing and expression were seen, as well as an increase in editing in the PFC in HRs relative to LRs.

Pattern of 5-HT$_{2C}$R editing and expression in the rat brain

Our study confirmed previous observations that, although both rat and human brain express multiple variants of 5-HT$_{2C}$R mRNA, the rat brain exhibits less diversity of mRNA variants [reviewed in (Werry et al., 2008)]. Indeed, compared with our recent 5-HT$_{2C}$R editing analysis in the human PFC (Dracheva et al., 2008c), fewer mRNA variants, and accordingly fewer protein isoforms, were detected in rats than in humans despite that fact that we assessed more clones in the rat than in the human study (5136 and 4709 clones, respectively). Of 32 possible mRNA variants, 27 were detected in the rat compared to 30 in the human. Accordingly, of 24 possible protein isoforms, 19 were predicted in the rat while 22 were predicted in the human. The pattern of editing was also different between humans and rats. In humans, *ABCD* is the most frequent mRNA variant in the whole brain (Niswender et al., 1999; Fitzgerald et al., 1999; Wang et al., 2000) and in the PFC (Dracheva et al., 2003; Dracheva et al., 2008c). This variant is edited at 4 sites and encodes the fully edited (compared to INI) VSV protein isoform (see Tables 2 and 3). In rats, *ABD* was the most frequently expressed variant in all brain regions assessed in the present study. This variant is edited at 3 sites and encodes the VNV isoform. Similar data have been reported for the whole rat brain (Burns et al., 1997; Niswender et al., 1999) and for the neocortex and amygdala in mice (Englander et al., 2005; Hackler et al., 2006).

Two factors could contribute to species-specific variations in the repertoire of 5-HT$_{2C}$R editing. First are differences in editing templates: A-to-I RNA editing is dependent on double stranded (ds) RNA, and the efficiency of editing is determined by the secondary structure of RNA (Valente and Nishikura, 2005). 5-HT$_{2C}$R region of editing forms a prerequisite double-stranded loop structure with the adjacent intron (Burns et al., 1997). Although the sequences of DNA encoding the putative stemloops are highly conserved between humans and rats (~ 88% identity), the predicted secondary structures of these loops differ significantly between the species (Werry et al., 2008), which may account for the

observed variations in editing patterns. Second, the editing process *per se* may differ between the species. For example, expression and/or activity of editing enzymes (ADAR1 and/or ADAR2) and expression of small nucleolar RNA or dsRNA helicase A (that were shown to influence 5-HT$_{2C}$R editing) (Yang et al., 2004; Vitali et al., 2005) may vary between humans and rats. The functional consequences of 5-HT$_{2C}$R editing differences between the species remain to be investigated.

Our study also confirmed the regional differences in 5-HT$_{2C}$R editing which were previously reported in human and rat (Werry et al., 2008). To our knowledge, 5-HT$_{2C}$R editing status in the VTA and NuAc has never been investigated. No differences between the PFC and VTA were detected in the expression of the four most frequently observed mRNA variants (*ABD, ABCD, AB, ABC*) or in the predicted protein isoforms (VNV, VSV, VNI, and VSI). However, the 5-HT$_{2C}$R was significantly more edited in the NuAc shell compared to both the PFC and the VTA. Specifically, an approximately 30% increase of the editing at D site as well as a modest increase at C site was observed in the NuAc shell compared to the PFC and VTA. Consequently, although the frequencies of the *ABD* mRNA variant and the VNV isoform (encoded by *ABD*) were only slightly increased, *ABCD* and VSV (encoded by *ABCD*) were ~ 60% higher in the NuAc shell compared to the other two regions, while the frequencies of the mRNA variants that are not edited at site D (*AB* and *ABC*) and, accordingly, the frequencies of the protein isoforms encoded by these variants (VNI and VSI, respectively) were ~60% lower in the NuAc shell.

The regional differences in 5-HT$_{2C}$R mRNA editing cannot be attributed to differences between the editing templates, as the sequence in the exon5-intron5 dsRNA stem-loop that is required for editing is identical in all regions of the rat brain. Thus, the higher 5-HT$_{2C}$R editing efficiency in the NuAc shell compared to the other regions could only result from regional differences in the editing process. Because the observed regional differences are largely determined by editing at D site, and because editing at this site is mostly catalyzed by ADAR2 (Werry et al., 2008), the simplest explanation for these regional differences is higher expression and/or higher activity of ADAR2 in the NuAc shell compared to the PFC and VTA. Indeed, our findings demonstrate higher expression of ADAR2 in the NuAc shell compared to the VTA and PFC. As discussed above, four different ADAR2 transcripts have been described, and those can be categorized into two categories. The first two transcripts (ADAR2f) encode functional ADAR2 proteins. The other two transcripts (ADAR2nonf) are generated by alterative splicing due to the ADAR2 auto-editing and encode truncated non-functional protein isoforms. In line with a current view that auto-editing of ADAR2 regulates its endogenous level (Feng et al., 2006), we detected a strong correlation between expression levels of ADAR2f and ADAR2nonf. Consequently, both types of ADAR2 transcripts showed increased expression in the NuAc shell compared to the VTA and PFC. In addition, although there were no correlations between ADAR2 expression and editing efficiencies at any site in any individual region, significant correlations were observed between ADAR2 (both ADAR2f and ADAR2nonf) and the editing at site D when the analysis was performed across all regions. This pattern suggests a direct association between ADAR2 transcript levels and the efficiency of editing at this site. ADAR2 expression levels

were also significantly higher in the VTA compared to the PFC. In line with that, a small but significant increase of editing at site D was detected in the VTA compared to the PFC.

A number of studies have suggested that while editing at D site requires ADAR2, editing at A and B sites is totally dependent on ADAR1 [reviewed in (Valente and Nishikura, 2005). Cross-talk between ADAR1 and ADAR2 activities has been observed, however, suggesting that relative expression of different ADARs may impact the editing efficiency at different sites (Valente and Nishikura, 2005). No correlations between ADAR1 expression and editing at A or B sites were detected in our study, implying that the level of ADAR1 mRNA expression did not limit the efficiency of editing at these sites. On the contrary, a significant correlation was observed between ADAR1 and editing at D site. Because ADAR1 does not catalyze D site editing, this correlation does not likely represent a functional association, but rather reflects strong correlations between ADAR1 and ADAR2 that were observed in our study. We also detected that the ADAR2f/ADAR1 ratio was significantly higher in the NuAc compared to the PFC and VTA (which did not differ from each other). Because ADAR1 has previously been shown to inhibit ADAR2 activity (Yang et al., 2004), the increased level of editing in the NuAc (mostly at site D) probably resulted not only from increased expression of ADAR2, but also from a higher ADAR2f/ADAR1 ratio in this region compared to PFC and VTA. These data clearly demonstrate that, as was suggested elsewhere (Werry et al., 2008), both absolute and relative levels of different ADARs are important in determining the identity and variety of the expressed 5-HT$_{2C}$R variants in different regions (and probably in different cell populations) in the brain.

Our data also indicate that the expression of ADAR2 variants and/or the ADAR2/ADAR1 ratio are imperfect predictors of editing at site D. Indeed, the observed correlations, albeit highly statistically significant, were moderate in magnitude (r ~ 0.7), suggesting that editing efficiency is only partially controlled by the mRNA expression of ADARs in the brain. Thus, other factors may contribute to regional differences in 5-HT$_{2C}$R editing. Because our regional dissections contained mixtures of different cell types, the functional association between ADAR2 and 5-HT$_{2C}$R editing is complicated by their cellular heterogeneity. ADAR2 is ubiquitously expressed and its expression may differ between different cell types. 5-HT$_{2C}$R expression, however, is restricted to specific cellular populations (see below).

Implications for 5-HT$_{2C}$R modulation of the DAergic neurotransmission

An important aspect of this study was the potential relevance of 5-HT$_{2C}$R mRNA editing to DA neurotransmission. Recent *in vivo* studies demonstrated that constitutive activity of 5-HT$_{2C}$Rs exerts tonic inhibitory control on DA release in the NuAc (De Deurwaerdere et al., 2004) underscoring the potential importance of ligand-independent 5-HT$_{2C}$R function in the modulation of reward circuitry (Berg et al., 2008). Previous studies conducted *in vitro* in transfected cells demonstrated that the unedited 5-HT$_{2C}$R possesses a significant degree of ligand-independent constitutive (or basal) activity, which is gradually diminished by editing (Niswender et al., 1999). Our data suggest that relative to the VTA, 5-HT$_{2C}$R mRNA editing in the NuAc shell is enhanced and, therefore, favors the receptor population with attenuated constitutive activity. This pattern is, however, the opposite of that previously hypothesized based on studies involving the intracranial microinjection of 5-HT$_{2C}$R inverse agonists to

the VTA and to the NuAc shell (Navailles et al., 2006; Navailles et al., 2008). Three considerations may reconcile our findings with these pharmacological data. First, 5-HT$_{2C}$R mRNA expression is approximately 3 times higher in the NuAc shell than in the VTA. Thus, despite the prevalence of the highly edited 5-HT$_{2C}$R isoforms in the NuAc shell, the overall constitutive activity may nevertheless be higher in the NuAc shell than in the VTA due to the absolute amount of less or unedited isoforms. Second, the 5-HT$_{2C}$R may be expressed by different cell types in the two regions. In the NuAc, 5-HT$_{2C}$R transcripts appear to localize primarily to medium-sized neurons that exhibit the distribution, localization, and morphology typical to striatal GABA efferent neurons (Eberle-Wang et al., 1997). Thus, 5-HT$_{2C}$Rs in the NuAc are likely to provide predominately negative feedback to the VTA DA neurons. Inhibition of constitutive 5-HT$_{2C}$R activity within the NuAc (by intracranial injection of an inverse agonist) would, therefore, increase DA release in this region (Navailles et al., 2006). In the VTA, recent studies localized the 5-HT$_{2C}$R protein to both GABA and DA neurons (Bubar and Cunningham, 2007). The 5-HT$_{2C}$R is excitatory (Stanford et al., 2005). Thus, its modulatory influence on DA neuron firing in the VTA (and, consequently, DA release in the NuAc) would reflect the functional balance of its opposing actions on GABA and DA neurons. Intra-VTA administration of an inverse agonist might, therefore, have no effect on DA release in the NuAc (Navailles et al., 2006). Third, recent work on mutant mice that solely express one of the two extreme editing isoforms of the receptor (INI or VGV) suggests that, as previously suggested by *in vitro* studies (Marion et al., 2004), the intracellular *vs.* cell surface localization of the receptor may differ between isoforms such that the ultimate functional impact (including the level of constitutive activity) of the receptors could not be unambiguously predicted by the patterns of 5-HT$_{2C}$R editing alone (Kawahara et al., 2008).

Region-specific differences in 5-HT$_{2C}$R editing between HRs and LRs

Despite the lack of differences in the expression of both 5-HT$_{2C}$R mRNA variants (5-HT$_{2C}$Rsp1 and 5-HT$_{2C}$Rsp2) between the behavioral phenotypes in the brain regions studied, 5-HT$_{2C}$R editing efficiency was higher in HRs *vs.* LRs in the PFC. Functional activity decreases with editing, thus this finding would predict reduced 5-HT$_{2C}$R function in the PFC of HRs that might be relevant for the individual differences in addiction vulnerability noted for this behavioral trait. The detected editing differences are consistent with the numerous accounts that central 5-HT$_{2C}$R exerts a negative control on the psychomotor-stimulant and rewarding effects of various drugs of abuse (see above).

The mechanisms and circuitry underlying the differences in the cortical 5-HT$_{2C}$R editing between HR and LR phenotypes are not easily understood, however, based on the current literature. Locomotor activity is associated with an increase in DA release in the NuAc (Dunnett and Robbins, 1992) and, compared to LRs, HRs have increased basal and stimulated DA levels in the NuAc (Hooks et al., 1992; Rouge-Pont et al., 1993). In the PFC, 5-HT$_{2C}$Rs are primarily localized to GABA interneurons (Liu et al., 2007) that are known to inhibit PFC glutamatergic pyramidal neurons that project to both the VTA and NuAc (Sesack and Carr, 2002). In the VTA these glutamatergic cortical afferents synapse on GABA (but not DA) mesoaccumbens neurons (Carr and Sesack, 2000). In the NuAc they also synapse on GABA neurons which, in turn, project to the VTA where they inhibit DA

neurons (Sesack and Pickel, 1992). Thus, by exciting the interposed GABA neurons it would be expected that the firing of the pyramidal PFC neurons would inhibit mesoaccumbens DA neurons. Consistent with the expected anatomical organization, several, but not all [see (Karreman and Moghaddam, 1996)], neurophysiological studies have shown that the firing of PFC glutamatergic neurons inhibits DA release in the NuAc (Jackson et al., 2001). Unless countered by other modulatory influences, stimulation of 5-HT$_{2C}$Rs in the PFC might, therefore, be expected to reduce glutamate output within the mesoaccumbens system leading to an increase of accumbal DA outflow. This increased DA release in the NuAc might then be expected to increase locomotion.

Our findings of enhanced editing (and presumably reduced function) of 5-HT$_{2C}$Rs in the PFC of HRs *vs.* LRs are at variance with the above speculation. Moreover, other studies that have evaluated the influence of PFC 5-HT$_{2C}$R function on the mesoaccumbens system have reported complex and seemingly contradictory results. Specifically, while an earlier study suggested that cortical 5-HT$_{2C}$Rs exert negative control over behavioral responses induced by cocaine (Filip and Cunningham, 2003), a recent study reported that those receptors enhance cocaine-evoked DA efflux in the NuAc (Leggio et al., 2008).

These apparent contradictions might be explained by the influence of cortical 5-HT$_{2C}$Rs on additional circuits that regulate accumbal DA release. Indeed, it is known that glutamatergic synapses of subcortical origin in the VTA significantly outnumber those of cortical origin (Omelchenko and Sesack, 2007). Moreover, some of these glutamatergic afferents to the VTA originate from other regions including the hypothalamus, thalamus and brainstem, which themselves receive PFC glutamatergic input (Sesack et al., 2003; Pinto et al., 2003; Geisler and Zahm, 2005; Omelchenko and Sesack, 2007; Geisler et al., 2007). Regulation of mesoaccumbens DAergic activity by the PFC may, therefore, involve polysynaptic glutamatergic inputs to the VTA from multiple sources. Thus, the control of the accumbal DA outflow by the PFC 5-HT$_{2C}$Rs may involve a functional balance between GABA and glutamate mediated influences. More research is needed to clarify the mechanisms and circuitry underlying this interaction.

Individual variation in PFC 5-HT$_{2C}$R editing (and, therefore, function) is likely to influence liability to a variety of psychiatrically relevant phenomena including substance abuse. Early studies detected reduced tissue content of 5-HT and its metabolite (5-HIAA) in the PFC and NuAc of HRs compared to LRs (Piazza et al., 1991). Lower cortical (but not accumbal) 5-HT was subsequently confirmed in HRs *vs.* LRs in (Thiel et al., 1999) suggesting decreased serotonergic function in the PFC of HRs. It is, therefore, tempting to speculate that the differences in the PFC 5-HT$_{2C}$R editing detected in the present study may stem from variations in the PFC 5-HT between the phenotypes. Interestingly, we recently detected increased 5-HT$_{2C}$R editing (manifested as increased frequency of the VSV isoform) in the PFC in suicide victims (Dracheva et al., 2008c). This difference was specific to suicide and was not associated with the comorbid psychiatric diagnoses (bipolar disorder or schizophrenia), demographic characteristics (e.g. age, sex) or prescribed psychoactive medications. The 5-HT$_{2C}$R editing differences in suicide had been previously suggested in other reports (Niswender et al., 2001; Gurevich et al., 2002). Of note, numerous studies suggested decreased serotonergic function in the brain of suicide victims, including 5-HT-

related abnormalities in the PFC (Mann, 2003). Accordingly, the enhanced level of the fully edited VSV isoform in the PFC (which is the most frequent 5-HT$_{2C}$R isoform in the human and one of the four most frequent isoforms in the rat PFC) may be associated with vulnerability to drug addiction and suicide. There is in fact a high degree of correlation between these disorders, and drug addiction is considered a risk factor for suicidal behavior (Arsenault-Lapierre et al., 2004). It is important to recognize, however, that the exact contribution of the PFC 5-HT$_{2C}$R editing to inter-individual vulnerability remains to be determined, since HRs differ from LRs not only in regard to reward sensitivity and goal-directed behavior (Marinelli, 2005), but also in their ability to learn operant tasks (Mitchell et al., 2005), as well as in their emotional reactivity (Stead et al., 2006). Specifically, HRs show less anxiety in novel contexts than LRs, which appears to be consistent with the differences in neuroendocrine function and in regulation of the serotonergic system between the phenotypes (Kabbaj et al., 2000; White et al., 2007; Mallo et al., 2008).

In summary, our study revealed distinct differences of 5-HT$_{2C}$R mRNA expression and editing among brain regions that comprise key components of the mesocorticolimbic system that imply significant differences in functional activity (including the levels of constitutive activity) among 5-HT$_{2C}$R neuronal populations within this circuitry. Moreover, the data indicate that these regional differences in editing may, at least in part, arise from the differences in expression levels of the editing enzyme, ADAR2. The discrete specificity of the 5-HT$_{2C}$R editing disturbance observed within the mesocorticolimbic neuronal populations between the HR and LR behavioral phenotypes significantly implicate the PFC in the pathophysiology of drug abuse liability.

ACKNOWLEDGMENTS

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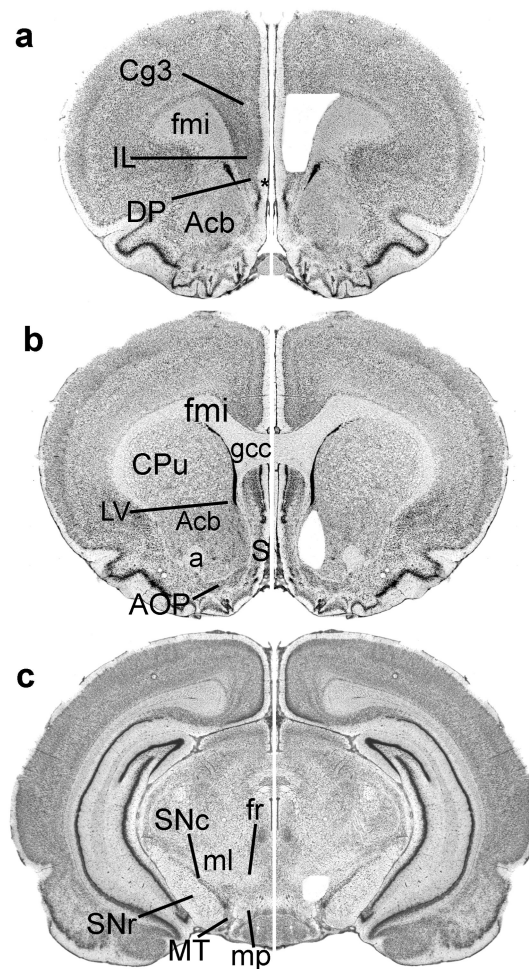
Fig. 1. Illustration of dissections

Twenty micron thick thionin stained sections before and after dissection of regions of interest. For photography, tissue was covered with 100% alcohol and temporarily cover-slipped. Only one hemisphere is shown before dissection and the mirror image of that hemisphere is shown after dissection. **a)** Level of the PFC dissection. The PFC dissections contained primarily **Cg3** and **IL**. **b)** Level of the NuAc dissection. The NuAc dissections were taken from its shell region at the level of the **gcc**. **c)** Level the VTA dissection. The VTA was dissected only at levels where the **fr** could be seen.

Abbreviations: **a**, anterior commissure, **Acb**, nucleus accumbens; **AOP**, accessory olfactory nucleus posterior; **Cg3**, cingulate cortex area 3; **DP**, dorsal peduncular cortex; **IL**, infralimbic cortex; **fmi,** forceps minor corpus callosum; **fr**, fasciculus retroflexus; **gcc**, genu corpus callosum; **LV**, lateral ventricle; **ml**, medial lemniscus; **mp**, mammillary peduncle; **MT**, medial terminal nucleus accessory optic tract; **S**, septal area; **SNc**, substantia nigra pars compacta; **SNr**, substantia nigra pars reticulate.
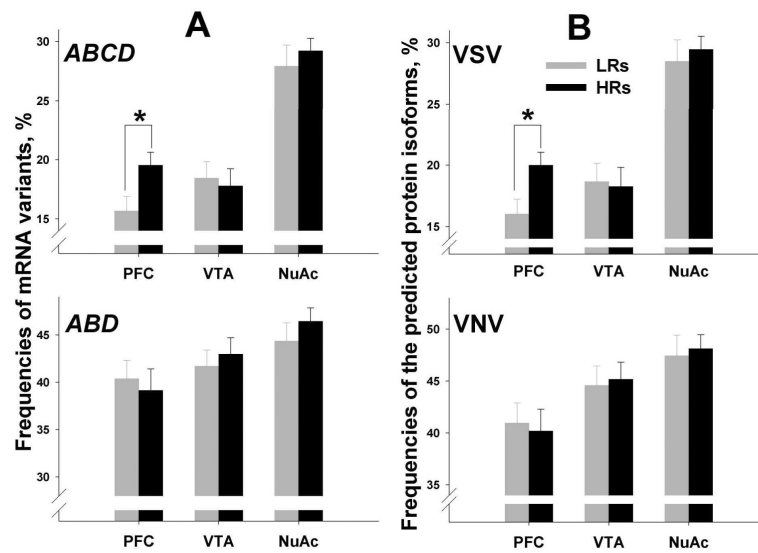
Fig. 2. Region-specific frequencies of the two most prevalent 5-HT$_{2C}$R mRNA variants (*ABD* and *ABCD*, panel A) and the two most prevalent 5-HT$_{2C}$R protein isoforms (VNV and VSV, panel B) in HRs and LRs

Shown are Means±SEM. *ABCD* and VSV were significantly increased in the PFC of HRs compared to LRs (*p<0.05)
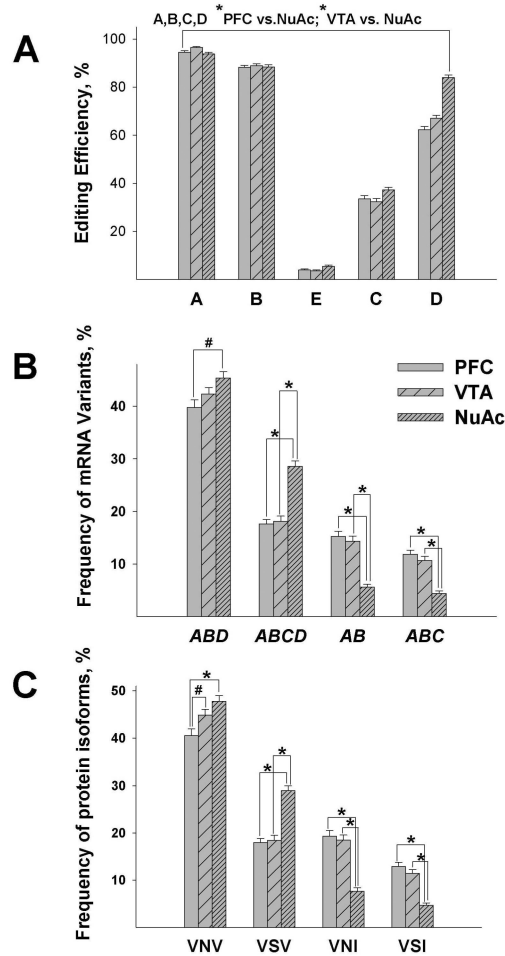
Fig. 3. Regional differences of 5-HT$_{2C}$R editing in the rat brain

Shown are Means±SEM of editing efficiencies at the five editing sites (panel A), frequencies of the mRNA variants (panel B), and frequencies of the predicted protein isoforms (panel C) in three different brain regions. All editing parameters are compared for the entire cohort (HRs and LRs combined). Shown are only the variants and isoforms that were observed at greater than ~ 5% frequencies. ($^{\#}$p<0.02; *p<0.0001).
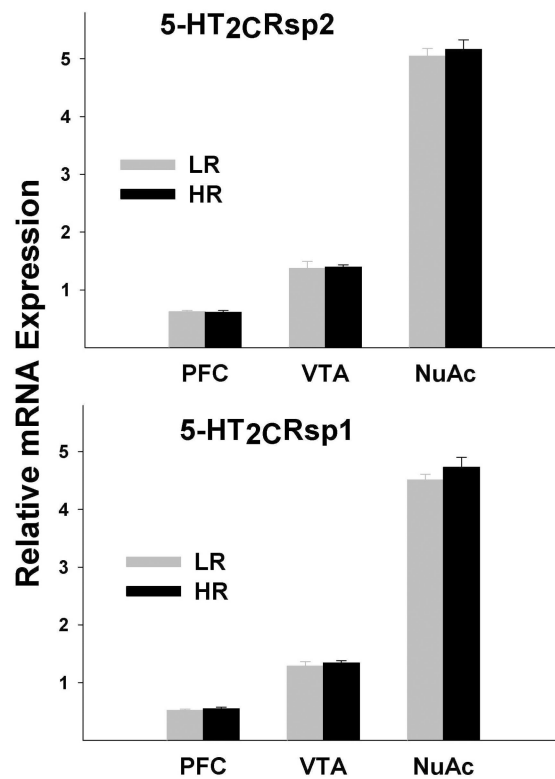
Fig. 4. Analysis of the 5-HT$_{2C}$R mRNA expression
mRNA expression was measured by qPCR. Shown are Means±SEM. 5-HT$_{2C}$Rsp2 and 5-HT$_{2C}$Rsp1 transcripts result from splicing at *sp2* and *sp1* sites and encode functional and truncated non-functional receptor, respectively. 5-HT$_{2C}$Rsp2 and 5-HT$_{2C}$Rsp1 expression levels were significantly different between pairs of regions (all p values <0.0001), but did not differ between HRs and LRs in any region.

Fig. 5. Analysis of the ADAR1 and ADAR2 mRNA expression
mRNA expression was measured by qPCR. Shown are Means±SEM. Two different TaqMan assays were employed to distinguish between the ADAR2 transcripts that encode functional (ADAR2f) and truncated non-functional (ADAR2nonf) proteins. ADAR1, ADAR2f, and ADAR2nonf expression levels were significantly different between pairs of regions (all p values <0.0001), but did not differ between HRs and LRs in any region.

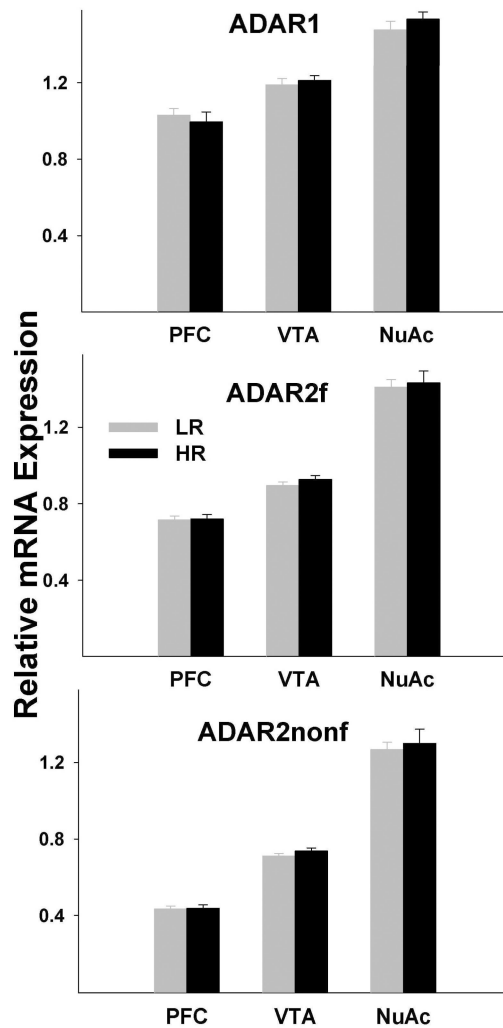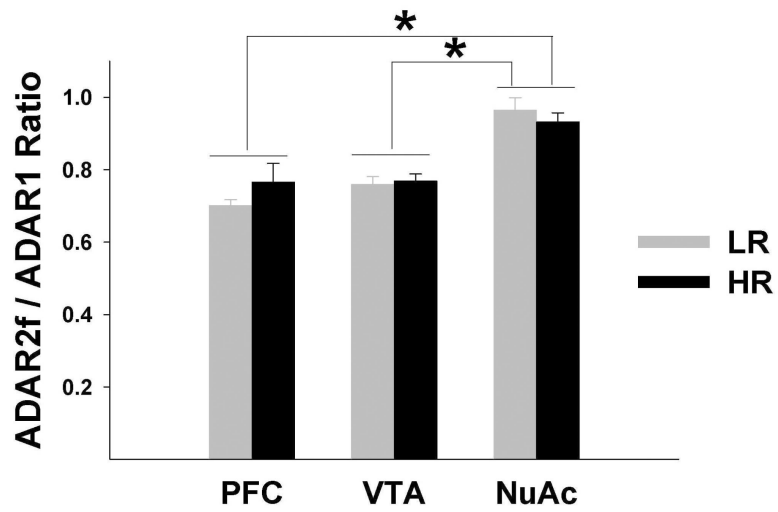**Fig. 6. Ratio between ADAR2f and ADAR1 expression levels**
Shown are Means±SEM. The ratios were significantly different between the NuAc shell and the two other regions (*p <0.0001), but did not differ between the PFC and VTA or between HRs and LRs in any region.

Table 1

Efficiency of editing at the five editing site (A, B, E, C, and D) of the 5-HT$_2$cR mRNA in the rat PFC, NuAc, and VTA

Editing Sites	PFC			VTA			NuAc		
	All rats N=38	LRs N=19	HRs N=19	All rats N=37	LRs N=18	HRs N=19	All rats N=37	LRs N=19	HRs N=18
A	94.6 ± 0.6%	* 93.1 ± 0.8%	96.1 ± 0.7%	96.5 ± 0.4%	96.6 ± 0.6%	96.4 ± 0.7%	93.9 ± 0.6%	93.9 ± 0.9%	93.9 ± 0.7%
B	88.2 ± 0.9%	* 86.9 ± 1.0%	89.6 ± 1.4%	88.9 ± 0.8%	89.5 ± 0.9%	88.4 ± 1.5%	88.4 ± 0.9%	86.6 ± 1.5%	90.3 ± 0.8%
E	3.9 ± 0.4%	4.4 ± 0.8%	3.5 ± 0.5%	3.5 ± 0.4%	4.5 ± 0.6%	2.5 ± 0.5%	5.4 ± 0.6%	5.6 ± 0.9%	5.1 ± 0.7%
C	33.5 ± 1.4%	* 31.6 ± 2.2%	35.3 ± 1.6%	32.3 ± 1.3%	32.6 ± 1.9%	32.1 ± 2.0%	37.2 ± 1.1%	36.2 ± 1.6%	38.3 ± 1.4%
D	62.3 ± 1.3%	* 60.8 ± 1.5%	63.8 ± 2.2%	67.1 ± 1.2%	68.1 ± 1.9%	66.3 ± 1.5%	84.0 ± 1.1%	83.0 ± 1.8%	85.0 ± 1.3%

The data represent Mean±SEM of the efficiencies in each study group. Editing efficiency at the A, B, C, and D sites (analyzed as repeated measures) was significantly lower in LRs compared to HRs in the PFC

*
(p<0.05)

Table 2

Frequencies for the observed 5-HT$_{2C}$R mRNA variants

mRNA variant	PFC				VTA			NuAc		
	$Human PFC; NC, N=35	All rats, N=38	LRs, N=19	HRs, N=19	All rats, N=37	LRs, N=18	HRs, N=19	All rats, N=37	LRs, N=19	HRs, N=18
ABD	15.9 ± 1.2%	39.7 ± 1.5%	40.4 ± 1.9%	39.1 ± 2.3%	42.3 ± 1.2%	43.0 ± 1.7%	41.7 ± 1.7%	45.4 ± 1.2%	44.4 ± 1.9%	46.4 ± 1.4%
ABCD	19.2 ± 1.2%	17.6 ± 0.9%	* 15.7 ± 1.2%	19.5 ± 1.1%	18.1 ± 1.0%	17.8 ± 1.5%	18.4 ± 1.4%	28.5 ± 1.0%	27.9 ± 1.8%	29.2 ± 1.1%
AB	2.7 ± 0.6%	15.2 ± 1.0%	15.1 ± 1.5%	15.4 ± 1.3%	14.3 ± 1.0%	13.4 ± 1.7%	15.2 ± 1.1%	5.6 ± 0.5%	5.6 ± 0.7%	5.6 ± 0.8%
ABC	2.9 ± 0.5%	11.8 ± 0.8%	11.5 ± 1.1%	12.1 ± 1.1%	10.6 ± 0.8%	11.2 ± 1.0%	10.1 ± 1.2%	4.4 ± 0.5%	3.8 ± 0.7%	5.0 ± 0.7%
A	6.6 ± 0.7%	4.1 ± 0.4%	4.2 ± 0.6%	3.9 ± 0.7%	4.2 ± 0.5%	4.0 ± 0.8%	4.3 ± 0.8%	2.1 ± 0.5%	2.9 ± 0.8%	1.2 ± 0.3%
NONE	8.7 ± 1.1%	2.6 ± 0.4%	3.2 ± 0.5%	2.0 ± 0.6%	0.9 ± 0.3%	0.6 ± 0.2%	1.2 ± 0.5%	1.9 ± 0.3%	2.3 ± 0.5%	1.5 ± 0.5%
ABED	1.9 ± 0.4%	1.2 ± 0.3%	1.5 ± 0.4%	0.9 ± 0.3%	0.8 ± 0.2%	1.2 ± 0.3%	0.5 ± 0.3%	2.5 ± 0.4%	2.6 ± 0.7%	2.4 ± 0.6%
AC	2.8 ± 0.5%	1.1 ± 0.3%	1.1 ± 0.4%	1.0 ± 0.4%	0.8 ± 0.2%	0.5 ± 0.2%	1.2 ± 0.3%	0.3 ± 0.1%	0.4 ± 0.2%	0.2 ± 0.2%
ABE	0.4 ± 0.2%	1.0 ± 0.3%	1.3 ± 0.4%	0.8 ± 0.3%	0.7 ± 0.2%	0.6 ± 0.2%	0.7 ± 0.3%	0.3 ± 0.1%	0.5 ± 0.2%	0.1 ± 0.1%
D	3.8 ± 0.6%	0.9 ± 0.2%	1.0 ± 0.3%	0.7 ± 0.3%	0.9 ± 0.3%	1.1 ± 0.4%	0.7 ± 0.3%	1.9 ± 0.3%	1.8 ± 0.6%	1.9 ± 0.4%
AD	6.4 ± 0.7%	0.8 ± 0.2%	0.6 ± 0.3%	1.0 ± 0.4%	2.6 ± 0.4%	2.2 ± 0.5%	2.9 ± 0.6%	2.4 ± 0.4%	3.1 ± 0.7%	1.7 ± 0.4%
C	2.1 ± 0.3%	0.6 ± 0.2%	0.9 ± 0.3%	0.3 ± 0.2%	0.5 ± 0.2%	0.5 ± 0.2%	0.5 ± 0.2%	0.4 ± 0.2%	0.5 ± 0.2%	0.4 ± 0.3%
CD	2.6 ± 0.5%	0.6 ± 0.2%	0.7 ± 0.3%	0.5 ± 0.3%	0.2 ± 0.1%	0.2 ± 0.2%	0.1 ± 0.1%	1.1 ± 0.2%	0.9 ± 0.3%	1.3 ± 0.4%
ABECD	3.9 ± 0.7%	0.6 ± 0.2%	0.2 ± 0.2%	0.9 ± 0.3%	0.9 ± 0.2%	1.2 ± 0.3%	0.6 ± 0.2%	1.1 ± 0.3%	1.3 ± 0.4%	0.9 ± 0.4%
ABEC	1.0 ± 0.3%	0.5 ± 0.2%	0.7 ± 0.3%	0.3 ± 0.2%	0.4 ± 0.1%	0.6 ± 0.2%	0.2 ± 0.2%	0.4 ± 0.2%	0.3 ± 0.2%	0.5 ± 0.3%
ACD	5.0 ± 0.7%	0.4 ± 0.1%	0.4 ± 0.2%	0.5 ± 0.2%	0.4 ± 0.1%	0.5 ± 0.2%	0.2 ± 0.2%	0.4 ± 0.1%	0.6 ± 0.2%	0.2 ± 0.2%
BD	0.7 ± 0.3%	0.4 ± 0.2%	0.5 ± 0.2%	0.3 ± 0.3%	0.5 ± 0.2%	0.2 ± 0.2%	0.7 ± 0.2%	0.1 ± 0.1%	0.0 ± 0.0%	0.1 ± 0.1%
AEC	3.5 ± 0.5%	0.3 ± 0.1%	0.5 ± 0.2%	0.1 ± 0.1%	0.1 ± 0.1%	0.0 ± 0.0%	0.2 ± 0.2%	0.3 ± 0.1%	0.3 ± 0.2%	0.2 ± 0.2%
AE	1.1 ± 0.3%	0.2 ± 0.1%	0.2 ± 0.2%	0.1 ± 0.1%	0.1 ± 0.1%	0.2 ± 0.2%	0.0 ± 0.0%	0.1 ± 0.1%	0.1 ± 0.1%	0.0 ± 0.0%
B	1.7 ± 0.4%	0.2 ± 0.1%	0.3 ± 0.2%	0.0 ± 0.0%	0.1 ± 0.1%	0.1 ± 0.1%	0.0 ± 0.0%	0.1 ± 0.1%	0.1 ± 0.1%	0.0 ± 0.0%
ED	0.2 ± 0.1%	0.1 ± 0.1%	0.0 ± 0.0%	0.1 ± 0.1%	0.2 ± 0.1%	0.4 ± 0.2%	0.1 ± 0.1%	0.3 ± 0.2%	0.1 ± 0.1%	0.5 ± 0.3%
AED	0.8 ± 0.2%	0.1 ± 0.1%	0.0 ± 0.0%	0.1 ± 0.1%	0.1 ± 0.1%	0.1 ± 0.1%	0.0 ± 0.0%	0.1 ± 0.1%	0.1 ± 0.1%	0.0 ± 0.0%
AECD	4.3 ± 0.6%	0.0 ± 0.0%	0.0 ± 0.0%	0.0 ± 0.0%	0.1 ± 0.1%	0.0 ± 0.0%	0.1 ± 0.1%	0.1 ± 0.1%	0.0 ± 0.0%	0.1 ± 0.1%
BCD	0.2 ± 0.1%	0.0 ± 0.0%	0.0 ± 0.0%	0.0 ± 0.0%	0.2 ± 0.1%	0.1 ± 0.1%	0.2 ± 0.2%	0.1 ± 0.1%	0.1 ± 0.1%	0.0 ± 0.0%

mRNA variant	PFC				VTA				NuAc			
	§Human PFC; NC, N=35	All rats, N=38	LRs, N=19	HRs, N=19	All rats, N=37	LRs, N=18	HRs, N=19	All rats, N=37	LRs, N=19	HRs, N=18	
E	0.6 ± 0.2%	0.0 ± 0.0%	0.0 ± 0.0%	0.0 ± 0.0%	0.1 ± 0.1%	0.1 ± 0.1%	0.0 ± 0.0%	0.2 ± 0.1%	0.1 ± 0.1%	0.2 ± 0.2%	
EC	0.5 ± 0.2%	0.0 ± 0.0%	0.0 ± 0.0%	0.0 ± 0.0%	0.1 ± 0.1%	0.0 ± 0.0%	0.1 ± 0.1%	0.0 ± 0.0%	0.0 ± 0.0%	0.0 ± 0.0%	
ECD	0.1 ± 0.1%	0.0 ± 0.0%	0.0 ± 0.0%	0.0 ± 0.0%	0.0 ± 0.0%	0.0 ± 0.0%	0.0 ± 0.0%	0.1 ± 0.1%	0.1 ± 0.1%	0.1 ± 0.1%	

The data represent Means±SEM of the frequencies in each study group. Only the mRNA variants that were detected at ~5% or greater frequency in at least one study group (highlighted) were analyzed.

§Editing frequencies in the human PFC of the psychiatrically normal control subjects (NC; N=35) are adopted from (Dracheva et al., 2008c). Editing frequency of *ABCD* was significantly higher in HR compared to LRs in the PFC

*
(p<0.05).

Table 3

Frequencies for the predicted 5-HT$_{2C}$R isoforms

Iso-form	PFC				VTA			NuAc		
	$Human PFC; NC N=35	All rats N=38	LRs N=19	HRs N=19	All rats N=37	LRs N=18	HRs N=19	All rats N=37	LRs N=19	HRs N=18
VNV	22.3 ± 1.28%	40.6 ± 1.4%	41.0 ± 1.9%	40.2 ± 2.1%	44.9 ± 1.2%	45.2 ± 1.7%	44.6 ± 1.9%	47.8 ± 1.2%	47.4 ± 2.0%	48.1 ± 1.4%
VNI	9.4 ± 0.80%	19.3 ± 1.2%	19.3 ± 1.7%	19.4 ± 1.7%	18.5 ± 1.1%	17.5 ± 1.7%	19.5 ± 1.3%	7.7 ± 0.7%	8.6 ± 1.1%	6.8 ± 0.8%
VSV	24.3 ± 1.10%	18.0 ± 0.9%	* 16.0 ± 1.2%	20.0 ± 1.1%	18.5 ± 1.0%	18.3 ± 1.6%	18.7 ± 1.5%	29.0 ± 1.0%	28.5 ± 1.8%	29.5 ± 1.1%
VSI	5.8 ± 0.62%	12.9 ± 0.9%	12.7 ± 1.2%	13.1 ± 1.3%	11.5 ± 0.8%	11.7 ± 1.0%	11.3 ± 1.3%	4.7 ± 0.5%	4.1 ± 0.7%	5.2 ± 0.7%
INI	8.7 ± 1.08%	2.6 ± 0.4%	3.2 ± 0.5%	2.0 ± 0.6%	0.9 ± 0.3%	0.6 ± 0.2%	1.2 ± 0.5%	1.9 ± 0.3%	2.3 ± 0.5%	1.5 ± 0.5%
VDV	2.7 ± 0.48%	1.3 ± 0.3%	1.5 ± 0.4%	1.0 ± 0.4%	0.9 ± 0.2%	1.3 ± 0.3%	0.5 ± 0.3%	2.6 ± 0.4%	2.7 ± 0.7%	2.4 ± 0.6%
VDI	1.4 ± 0.37%	1.2 ± 0.3%	1.5 ± 0.4%	0.9 ± 0.3%	0.8 ± 0.2%	0.8 ± 0.3%	0.7 ± 0.3%	0.4 ± 0.2%	0.6 ± 0.3%	0.1 ± 0.1%
INV	3.8 ± 0.59%	0.9 ± 0.2%	1.0 ± 0.3%	0.7 ± 0.3%	0.9 ± 0.3%	1.1 ± 0.4%	0.7 ± 0.3%	1.9 ± 0.3%	1.8 ± 0.6%	1.9 ± 0.4%
VGI	4.5 ± 0.61%	0.8 ± 0.3%	1.1 ± 0.4%	0.5 ± 0.3%	0.5 ± 0.2%	0.6 ± 0.2%	0.5 ± 0.2%	0.7 ± 0.2%	0.7 ± 0.2%	0.7 ± 0.3%
ISI	2.1 ± 0.32%	0.6 ± 0.2%	0.9 ± 0.3%	0.3 ± 0.2%	0.5 ± 0.1%	0.5 ± 0.2%	0.5 ± 0.2%	0.4 ± 0.2%	0.5 ± 0.2%	0.4 ± 0.3%
ISV	2.6 ± 0.46%	0.6 ± 0.2%	0.7 ± 0.3%	0.5 ± 0.3%	0.2 ± 0.1%	0.2 ± 0.2%	0.1 ± 0.1%	1.1 ± 0.2%	0.9 ± 0.3%	1.3 ± 0.4%
VGV	8.2 ± 0.88%	0.6 ± 0.2%	0.2 ± 0.2%	0.9 ± 0.3%	0.9 ± 0.2%	1.2 ± 0.3%	0.7 ± 0.2%	1.2 ± 0.3%	1.3 ± 0.4%	1.1 ± 0.4%
MNV	0.7 ± 0.29%	0.4 ± 0.2%	0.5 ± 0.2%	0.3 ± 0.3%	0.5 ± 0.1%	0.2 ± 0.2%	0.7 ± 0.2%	0.1 ± 0.1%	0.0 ± 0.0%	0.1 ± 0.1%
MNI	1.7 ± 0.40%	0.2 ± 0.1%	0.3 ± 0.2%	0.0 ± 0.0%	0.1 ± 0.1%	0.1 ± 0.1%	0.0 ± 0.0%	0.1 ± 0.1%	0.1 ± 0.1%	0.0 ± 0.0%
IDV	0.2 ± 0.14%	0.1 ± 0.1%	0.0 ± 0.0%	0.1 ± 0.1%	0.2 ± 0.1%	0.4 ± 0.2%	0.1 ± 0.1%	0.3 ± 0.2%	0.1 ± 0.1%	0.5 ± 0.3%
IDI	0.6 ± 0.21%	0.0 ± 0.0%	0.0 ± 0.0%	0.0 ± 0.0%	0.1 ± 0.1%	0.1 ± 0.1%	0.0 ± 0.0%	0.2 ± 0.1%	0.1 ± 0.1%	0.2 ± 0.2%
IGI	0.5 ± 0.18%	0.0 ± 0.0%	0.0 ± 0.0%	0.0 ± 0.0%	0.1 ± 0.1%	0.0 ± 0.0%	0.1 ± 0.1%	0.0 ± 0.0%	0.0 ± 0.0%	0.0 ± 0.0%
IGV	0.1 ± 0.06%	0.0 ± 0.0%	0.0 ± 0.0%	0.0 ± 0.0%	0.0 ± 0.0%	0.0 ± 0.0%	0.0 ± 0.0%	0.1 ± 0.1%	0.1 ± 0.1%	0.1 ± 0.1%
MSV	0.2 ± 0.11%	0.0 ± 0.0%	0.0 ± 0.0%	0.0 ± 0.0%	0.2 ± 0.1%	0.1 ± 0.1%	0.2 ± 0.2%	0.1 ± 0.1%	0.1 ± 0.1%	0.0 ± 0.0%

The data represent Means±SEM of the frequencies in each study group. Only the isoforms that had 5% or greater frequency in at least one study group (highlighted) were analyzed.

$Editing frequencies in the human PFC of the psychiatrically normal control subjects (NC; N=35) are adopted from (Dracheva et al., 2008c). Editing frequency of VSV was significantly higher in HR compared to LRs in the PFC

*
(p<0.05).

Table 4

Correlation between editing efficiencies and ADARs' mRNA expression. Analysis was performed across three brain regions (PFC, VTA, NuAc) combined (N=112). Significant correlations (p<0.0012) are shown in "Bold".

Editing Efficiency or mRNA expression	ADAR1	ADAR2f	ADAR2nonf
A	r=-0.138 p=0.147	r=-0.134 p=0.164	r=-0.087 p=0.366
B	r=-0.070 p=0.466	r=-0.001 p=0.991	r=0.032 p=0.737
E	r=0.179 p=0.062	r=0.2761 p=0.004	r=0.244 p=0.010
C	r=0.149 p=0.112	r=0.160 p=0.096	r=0.170 p=0.076
D	**r=0.540 p<0001**	**r=0.685 p<0001**	**r=0.712 p<0001**
ADAR1		**r=0.862 p<0001**	**r=0.823 p<0001**
ADAR2f			**r=0.931 p<0001**