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5-HT_{2C} receptor RNA editing in the amygdala of C57BL/6J, DBA/2J, and BALB/cJ mice

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Abstract

Post-transcriptional RNA editing of the G-protein coupled 5-hydroxytryptamine-2C (5-HT_{2C}) receptor predicts an array of 24 receptor isoforms, some of which are characterized by reduced constitutive activity and potency to initiate intracellular signaling. The amygdala is integral to anxiety, fear, and related psychiatric diseases. Activation of 5-HT_{2C} receptors within the amygdala is anxiogenic. Here, we describe the RNA editing profiles from amygdala of two inbred mouse strains (BALB/cJ and DBA/2J) known to be more anxious than a third (C57BL/6J). We confirmed the strain anxiety differences using light ↔ dark exploration, and we discovered that BALB/cJ and DBA/2J are each characterized by a higher functioning RNA editing profile than C57BL/6J. BALB/cJ and DBA/2J exhibit a roughly two-fold reduction in C site editing, and a corresponding two-fold reduction in the edited isoform VSV. C57BL/6J is characterized by a relative decrease in the unedited highly functional isoform INI. We estimated the heritability of editing at the C site to be approximately 40%. By sequencing genomic DNA, we found complete conservation between C57BL/6J, BALB/cJ, DBA/2J and 37 other inbred strains for the RNA edited region of *Htr2c*, suggesting *Htr2c* DNA sequence does not influence variation in *Htr2c* RNA editing between inbred strains of mice. We did, however, discover that serotonin turnover is reduced in BALB/cJ and DBA/2J, consistent with emerging evidence that synaptic serotonin levels regulate RNA editing. These results encourage further study of the causes and consequences of 5-HT_{2C} receptor RNA editing in the amygdala of mice.

Keywords: RNA editing; 5-HT_{2C} receptor; *Htr2c*; Amygdala; Anxiety; Fear; Inbred mouse strain; Serotonin

1. Introduction

The complexity of the contributions of the 5-HT_{2C} receptor to brain function is increased by post-transcriptional modifications of receptor RNA prior to translation to receptor protein. In a process termed 'RNA editing', the potential for 32 RNA isoforms and 24 protein isoforms of the 5-HT_{2C} receptor is realized by enzymatic adenosine to inosine conversions at five nucleotide positions (termed A, B, E, C, and D) in the 5th exon corresponding to the second intracellular loop of the receptor (Sanders-Bush et al., 2003; Schmauss, 2005). Importantly, in vitro studies have characterized dramatically altered function in RNA edited variants of the 5-HT_{2C} receptor when the C site has been edited. For example, the edited isoform VSV has four-fold reduced constitutive activity and four to five-fold reduced potency to initiate intracellular signals relative to the unedited isoform INI (Niswender et al., 1999; Wang et al., 2000; see also Burns et al., 1997; Fitzgerald et al., 1999; Herrick-Davis et al., 1999; Price and Sanders-Bush, 2000; Visiers et al., 2001; Berg et al., 2001; Price et al., 2001; McGrew et al., 2004).

While editing of *Htr2c* (the 5-HT_{2C} receptor gene) RNA was first reported in 1997 (Burns et al., 1997), and has been repeatedly confirmed, only a few recent studies have examined regulation of the process using animal models (Gurevich et al., 2002a,b; Englander et al., 2005; Iwamoto et al., 2005; Sodhi et al., 2005). These studies circumscribe 5-HT_{2C} receptor RNA editing as a dynamic process that is responsive to environmental challenges, including stress (Englander et al., 2005), behavioral models of depression (Iwamoto et al., 2005), 5-HT

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enhancement or depletion (Gurevich et al., 2002a,b), and psychoactive medications, such as antidepressants (Englander et al., 2005; Iwamoto et al., 2005) and antipsychotics (Sodhi et al., 2005). RNA editing has been suggested to be a mechanism allowing 5-HT_{2C} receptors to adjust to changing circuit activity, perhaps to maintain optimal response properties (Gurevich et al., 2002a,b).

In humans, changes in *Htr2c* RNA editing have been found post-mortem in the prefrontal cortex of depressed suicide victims (Niswender et al., 2001; Gurevich et al., 2002b; Iwamoto and Kato, 2003) as well as in the prefrontal cortex of persons with schizophrenia (Sodhi et al., 2001; but see Niswender et al., 2001; Dracheva et al., 2003; Iwamoto and Kato, 2003). To date, the most consistent changes in RNA editing with psychiatric disease have come from studies of depression and suicide. The data of Gurevich et al. (2002b) show a pattern in which depressed suicide victims have increased C and E site editing but decreased D site editing. Relative to matched controls, post-mortem prefrontal cortex thus had an increased pool of 5-HT_{2C} mRNA encoding receptors with reduced function. In a recent review, Schmauss suggests that changes in 5-HT_{2C} pre-RNA editing found in major depression reflect a previously unrecognized molecular response to stress that can be prevented by chronic antidepressant treatment (Schmauss, 2005). Although this hypothesis is difficult to test in humans, results from a combination of forced swim test and antidepressant treatment in mice (Englander et al., 2005) and learned helplessness and antidepressant treatment in rats (Iwamoto et al., 2005) provide credibility. In both studies, the stress manipulation caused *Htr2c* RNA editing changes in neocortex that were ameliorated by antidepressant treatment. Thus, although our understanding of the in vivo causes and consequences of variation in RNA edited profiles of the 5-HT_{2C} receptor is in its infancy, there is already significant promise that *Htr2c* RNA editing represents a novel molecular mechanism of interest to mental health.

The amygdaloid complex plays important roles in anxiety, fear, and related psychiatric disorders (Sah et al., 2003; Miller et al., 2005; Ballenger et al., 2004; Nutt et al., 1998; Sturm et al., 2003; Villafuerte and Burmeister, 2003). Although not completely understood, part of the mechanisms underlying these roles lie with the neurotransmitter serotonin, its transporter, and several of its cognate G-protein coupled receptors, including 5-HT_{2C} (Holmes and Hariri, 2003; Hariri and Weinberger, 2003). 5-HT_{2C} receptors are present in areas known to be involved in anxiety states, including the amygdala (Pompeiano et al., 1994), and 5-HT_{2C} receptor activation increases anxiety-like behaviors. For example, the non-selective 5-HT_{2C} receptor agonist *m*-chlorophenylpiperazine (mCPP) elicits anxiety in both humans and animals (Gatch, 2003). Direct infusion of mCPP into the amygdala of rats potentiates innate fear-like responses in open field measures (Campbell and Merchant, 2003). More recently, infusion of the preferential 5-HT_{2C} receptor agonist 6-chloro-2 [1ppiperazinyl] pyrazine (MK-212) into the amygdala of rats has also been shown to increase anxiety-like responses (de Mello Cruz et al., 2005). Between inbred strains of mice, substantial differences have been demonstrated in serotonin receptor binding site density within the amygdala (Yilmazer-Hanke et al., 2003). This variation correlated with fear-sensitized acoustic startle responses, although the methods used for binding site density precluded statements about specific serotonin receptor subtypes.

To date, there has been no study of *Htr2c* RNA editing in amygdala, despite the role of 5-HT_{2C} receptors in amygdala function and the role of amygdala function in psychiatric disease. Here, we have tested for differences in *Htr2c* RNA editing within the amygdala of a low anxious mouse model, the inbred strain C57BL/6J, and two high anxious mouse models, the inbred strains BALB/cJ and DBA/2J (Crawley et al., 1997; Lamberty, 1998; Boukwknecht et al., 2000; Cook et al., 2001; Tarantino et al., 2000; Holmes et al., 2002; Lipkind et al., 2004; Yilmazer-Hanke et al., 2003; Dulawa et al., 2004; Griebel et al., 2000; Falls et al., 1997). We confirmed these reported anxiety differences using a standard anxiety test, the light ↔ dark exploration box (File, 2001; File et al., 2004). In addition, we sequenced *Htr2c* in the region that could interfere with RNA editing mechanisms and thus underlie strain differences, and we assayed 5-HT and its metabolite 5-HIAA (5-hydroxyindolacetic acid) from amygdala, as serotonin has been implicated in RNA editing differences, as well as anxiety, fear, and related psychiatric pathophysiology.

2. Methods

2.1. Htr2c RNA editing

Eight young adult (8–12 weeks of age) male mice from each strain (BALB/cJ, C57BL/6J, and DBA/2J) were killed by cervical dislocation, their brains rapidly removed from the skull, placed in a mouse brain blocking matrice (catalog #51386, www.stoelting.com), and two 1 mm thick coronal sections encompassing the anterior–posterior extent of the amygdala were dissected using ventral surface landmarks (posterior to the optic chiasm and anterior to the pons). From the coronal sections, cortical tissue containing visible amygdala nuclei was dissected en masse using the rhinal sulcus as a guide. All mice were housed on a 12 h:12 h light:dark cycle prior to dissection. Separate cohorts of mice were used for measures of RNA editing and for behavior and biogenic amine quantification. This produced measures of RNA editing that were unbiased by the behavioral tests and therefore representative of trait rather than state anxiety levels.

Amygdala samples were homogenized in TriReagent (Sigma–Aldrich) before RNA extraction. Total RNA (2 μg) was extracted by standard methods and utilized for RT-PCR generation of 5-HT_{2C} receptor as described previously (Sodhi et al., 2005) using primers that spanned the edited region (sense 5′-TTTCAACTGCGTCCATCATGCACCT-3′ and antisense 5′-AACGAAGTT-GGGGTCATTGAGCACGCAC-3′). PCR products were ligated and cloned into the pGEM-T vector (Promega) for bacterial transformation. Bacterial colonies generated were randomly chosen for characterization as follows. Bacteria from each colony, which correspond to single mRNA transcripts, were directly inoculated in a PCR reaction mixture containing 0.2 μM sense oligonucleotide primer (5′-ATATCGCTGGATCGGTATGTAG-3′) and 0.2 μM of an antisense biotinylated primer (5′-BIOTIN-CGAATTGAAACGGC-TATGCT-3′), 1× Taq Gold buffer with 1.5 mM $MgCl_2$ (Perkin-Elmer), 0.75 U Taq Gold (Perkin-Elmer) and 0.24 mM dNTPs. Samples were heated to 96 °C for 5 min and then amplified for 45 cycles each consisting of 20 s at 96 °C, 30 s at 58 °C and 20 s at 72 °C in a thermal cycler (Peltier, MJ Research) resulting in a 60 bp product in 30 μl total reaction volume. Five microlitres were resolved by 3% agarose gel electrophoresis, with a 20 bp DNA standard (Qiagen) to ensure successful transformation. The remainder of the PCR product was used in the subsequent assay. Pyrosequencing (Biotage.com,

Uppsala, Sweden) was carried out using 10 μM of the sense primer (5′-ATATCGCTGGATCGGTATGTAG-3′) and the dispensation sequence 5′-GC AGCT AGTCGT AGAGTCT AGCT-3′.

To achieve adequate statistical power (Sodhi et al., 2005), 850 Pyrograms (mean of 35 RNA isolates per each of eight animals per strain) were scored for the *Htr2c* RNA editing sites A, B, E, C, and D as a string of five 0 s (unedited) and 1 s (edited). These data were recoded to several dependent variables using a Stata script (www.stata.com). These variables included frequency of editing at the individual editing sites (e.g. C), the frequency of RNA isoforms (e.g. ABCD), and the frequency of the predicted 5-HT_{2C} receptor isoforms (e.g. INI, VSV). Pyrosequencing of samples was performed in the Neurogenomics Core Laboratory in the Center for Molecular Neuroscience at Vanderbilt University Medical Center.

2.2. DNA sequencing

DNA from 40 inbred strains representative of the Mouse Phenome Database (http://www.jax.org/phenome) was purchased from Jackson Laboratories (http://www.jax.org/dnares). The genomic region of the *Htr2c* gene involved in RNA editing was amplified by PCR and sequenced to test for variation that might underlie strain differences. PCR sense and antisense primer sequences were 5′-TATTTGTGCCCCGTCTGG-3′ and 5′-GTCATAGCATTTTGTATT-CAGTGTTGC-3′, respectively. These sequences amplify a 458 bp fragment ranging from the 26th base of *Htr2c* exon 5 to beyond the entire exon/intron duplex region. Each PCR reaction consisted of 1× Applied Biosystems PCR Buffer II, 125 μM dNTP, 0.2 μM forward primer, 0.2 μM reverse primer, 1.5 U Taq Gold, 2.0 mM magnesium chloride, 25 ng template, PCR H_2 0–50 μl. The PCR reaction steps were 95° for 5 min, 94° for 30 s, 54° for 30 s, 72° for 90 s, repetition of steps 2–4 34 more times, and 72° for 5 min. Part of the PCR product was checked for purity and approximate size by gel electrophoresis with a 100 bp DNA standard (Qiagen), and the remainder was cleaned via QIAquick PCR purification kit according to the manufacturer's directions. The amplicon was then sequenced in both forward and reverse directions using the PCR primers as sequencing primers, using BigDye Terminator chemistry and an ABI 3730 × l DNA Analyzer. DNA sequences were scored using Applied Biosystems Sequencing Analysis Software v5.2. Sequence files were aligned using ClustalW software (http://www.ebi.ac.uk/clustalw/) and manually inspected for sequence variation with reference to C57BL/6J. Mouse strains sequenced included: 129S1/SvlmJ, A/J, AKR/J, BALB/cByJ, BTBR T + tf/tf, BUB/BnJ, C3H/HeJ, C57BL/10J, C57BL/6J, C57BLKS/J, C57BR/cdJ, C57L/J, C58/J, CAST/Ei, CBA/J, CE/J, CZECHII/Ei, DBA/2J, FVB/NJ, I/LnJ, JF1/Ms, KK/HlJ, LP/J, MA/MyJ, MOLF/Ei, MSM/Ms, NOD/LtJ, NON/LtJ, NZB/BlNJ, NZW/LacJ, PERA/Ei, PL/J, PWK/Ph, RIIIS/J, SEA/GnJ, SJL/J, SM/J, SPRET/Ei, SWR/J, and WSB/Ei. DNA sequencing was performed in the Vanderbilt DNA Sequencing Facility at Vanderbilt University Medical Center (http://seq.mc.vanderbilt.edu/DNA/html/index.html).

2.3. 5-HT and 5-HIAA

Bilateral amygdala samples from the brains of 10 male, 8–12 week old mice per strain were rapidly dissected as above and frozen on dry ice. The tissue was homogenized in 100–750 μl of 0.1 M TCA, which contains 10^{-2} M sodium acetate, 10^{-4} M EDTA and 10.5% methanol (pH 3.8). Samples were spun in a microcentrifuge at 10,000 × *g* for 20 min. The supernatant was removed and stored at −80° (Cransac et al., 1996). The pellet was saved for protein analysis. Supernatant was thawed and spun for 20 min. Samples of the supernatant were then analyzed for the biogenic monoamines 5-HT (serotonin) and 5-HIAA (5-hydroxyindoleacetic acid).

Biogenic amines were determined by a specific HPLC assay utilizing an Antec Decade (oxidation: 0.7) electrochemical detector. Twenty microlitres samples of the supernatant were injected using a Water 717+ autosampler onto a Waters Nova-Pak C18 HPLC column. Biogenic amines were eluted with a mobile phase consisting of 89.5% 0.1 M TCA, 10^{-2} M sodium acetate, 10^{-4} M EDTA and 10.5% methanol (pH 3.8). Solvent was delivered at 0.7 ml/min using a Waters 515 HPLC pump. Using this HPLC solvent the following biogenic amines elute in the order: noradrenaline, MHPG, adrenaline, DOPAC, Dopamine, 5-HIAA, HVA, 5-HT, and 3-MT (Lindsey et al., 1998). HPLC control and data acquisition were managed by Millennium 32 software. 5-HT and 5-HIAA levels are expressed in picogram per milligrams of protein (pg/mg protein).

Total protein concentration of the brain extracts was determined using BCA Protein Assay Kit purchase from Pierce Chemical Company (Rockford, IL). Bioamine assays were performed by the Neurochemistry Analytical Core Laboratory in the Center for Molecular Neuroscience at Vanderbilt University Medical Center.

2.4. Anxiety behavior

Ten male young adult mice (8–12 weeks of age) for each strain (BALB/cJ, C57BL/6J, DBA/2J) were tested for anxiety measures using the light ↔ dark exploration with careful attention to procedural details outlined by File et al. (2004). All mice were run on the same day during daylight hours and the light phase of the animals' housing cycle. The order of testing for the animals was random with regard to strain. The light ↔ dark exploration test session for each mouse was a standard 5 min. Equipment consisted of eight open field chambers with dark box inserts integrated by a PC running Activity Monitor software version 5 (Med-Associates Inc., www.med-associates.com). Open field chambers were cleaned between test sessions, and overhead lights were on. Using the Activity Monitor software to divide the chamber into two zones matching the physical space of the white and dark sides, three measures of anxiety were calculated. Summary statistics for the three anxiety measures (''transitions'', ''latency'', and ''time'') were exported to a delimited ASCII file and imported for further analysis in Stata 9 statistical software. The number of transitions was the sum of movements of the mouse between the white and black sides of the chamber over the test session. Latency was the time for the mouse to first enter the black side after its initial placement in the white side facing away from the dark side. Time on the white side was the total time the mouse spent on that side during the test session. Transitions and time are inversely proportional to increasing anxiety while latency is proportional to increasing anxiety. Non-parametric Kruskal–Wallis tests were used to test strain differences. One-tailed significance criteria were used with comparisons to C57BL/6J, because of prior evidence that C57BL/6J was the less anxious strain. All behavior tests were performed in the Vanderbilt Murine Neurobehavioral Lab shared behavior core facilities at Vanderbilt University Medical Center (https://medschool.mc.vanderbilt.edu/mnl).

2.5. Statistics

All statistical analyses were performed using Stata statistical software (version 9.1, www.stata.com). Strain differences in anxiety behavior or bioamine levels were tested by Kruskal–Wallis tests or ANOVAs. With the *Htr2c* RNA editing data left in its raw form (e.g. 0 and 1 s for ''no C edit'' versus ''C edit'' or ''not isoform VSV'' versus ''isoform VSV''), strain differences in *Htr2c* RNA editing were tested using parametric generalized estimating equations (GEE) with binomial family, logit link, and exchangeable correlation settings. GEE accounts for the added complication of repeated binary scores from the same mouse, which give rise to clustered data (see chapter 31 of Kirkwood and Sterne (2003) for an excellent discussion of clustered data sets, and chapter 8 in Vittinghoff et al. (2005) for use of Stata to analyze repeated measures and clustered data sets).

To assess strength of genetic determination of variation in the amounts of C site editing and the isoform VSV, we used (1) the formula of Hegmann and Possidente (1981) to estimate narrow sense heritability from inbred strains, 0.5 × (additive genetic variance)/((0.5 × (additive genetic variance)) + (environmental variance)), and (2) the Omega squared statistic from a one-way ANOVA for strain differences to estimate broad sense heritability, using the omnibus test from the ANOVA as the test for significant genetic determination. Because inbred strains are isogenic, the average of the variances within lines is an estimate of environmental variance. The variance of strain means is an estimate of additive genetic variance. Since *Htr2c* RNA editing data on the observed scale is categorical, to estimate heritability on a numeric scale, each animal's data were transformed to a percentage summary statistic, and genetic variance and environmental variance calculated from the distribution of animal summary statistics. Heritability of the amount of INI was not calculated due to substantial skew in the distribution (many animals exhibited 0% or close to 0% INI).

2.6. Animals

All experimental procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health (publication 86–23) and the Vanderbilt University Animal Care and Use Committee.

3. Results

3.1. Anxiety behavior

Anxiety differences between C57BL/6J and both BALB/cJ and DBA/2J were confirmed in the Light ↔ Dark Box (Fig. 1). The number of transitions between the white and black sides of the chamber over the test session was greatest in C57BL/6J followed by BALB/cJ and DBA/2J. A non-parametric Kruskal–Wallis test determined significant strain differences (χ^2(2 d.f.) = 10.261, $p = 0.006$). Post hoc Wilcoxon rank-sum tests revealed significant differences between both C57BL/6J and DBA/2J ($z = 3.384$, $p = 0.0004$) and C57BL/6J and BALB/cJ ($z = 1.742$, $p = 0.04$). BALB/cJ was not different than DBA/2J ($z = 0.948$, $p = 0.34$). The time during the test session spent on the white side of the test chamber was higher in C57BL/6J and BALB/cJ than in DBA/2J. Strain differences were significant, using a Kruskal–Wallis test (χ^2(2 d.f.) = 5.770, $p = 0.028$). Post hoc pairwise Wilcoxon rank-sum tests revealed a significant difference between C57BL/6J and DBA/2J ($z = 2.570$, $p = 0.005$). Latency to first enter the black side of the test chamber was most rapid in C57BL/6J, followed by DBA/2J, and then BALB/cJ. Strain differences tested as significantly different using a Kruskal–Wallis test (χ^2(2 d.f.) = 4.98, $p = 0.04$). Post hoc pairwise Wilcoxon rank-sum tests revealed a significant difference between C57BL/6J and BALB/cJ ($z = 2.041$, $p = 0.02$).

3.2. Htr2c RNA editing

Inbred strains BALB/cJ, C57BL/6J, and DBA/2J varied substantially in *Htr2c* RNA editing profiles in amygdala (Tables 1–3). An approximate two-fold difference in percentage editing was observed at site C, with increased editing in strain C57BL/6J relative to BALB/cJ and DBA/2J (Table 1). Virtually no editing was found at site E. Of 32 possible RNA isoforms, we found evidence of 22, and only five of these were also observed at greater than 5% relative frequency (Table 2). Of 24 predicted protein isoforms, we observed RNA evidence for only 16, and we observed only five for which at least one of the strains had 5% or greater relative frequency (Table 3). These were INI, VNI, VNV, VSI, and VSV. The isoform VNV was the most prevalent, followed by VSV. VSV followed the differences in C site editing, again with an approximate two-fold difference in the percentage editing in C57BL/6J relative to BALB/cJ and DBA/2J.

Table 1
Edit site percentages

Site	BALB/cJ	C57BL/6J	DBA/2J
A	94	96	82
B	89	94	80
E	4	0	2
C	21	43	28
D	79	83	64

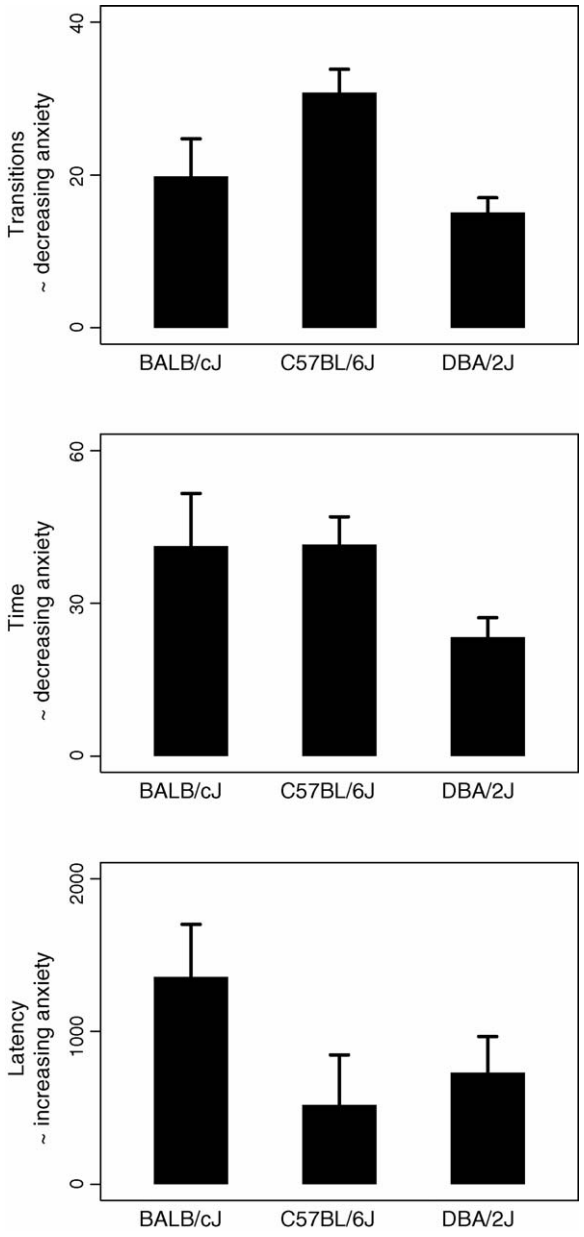

Fig. 1. Light ↔ dark exploration test. The data are presented as strain means with standard errors. The top panel displays the number of transitions between the white and black sides of the chamber over the test session. This is proportional to decreasing anxiety. The middle panel displays the time during the test session spent on the white side of the test chamber. This is proportional to decreasing anxiety. The bottom panel displays the latency to first enter the black side of the test chamber. This is proportional to increasing anxiety.

GEE models for correlated binary dependent variables confirmed the statistical significance of the differences between strains in frequency of editing at site C (omnibus Wald χ^2(2 d.f.) = 19.76, $p < 0.0001$) and in the isoform VSV (omnibus Wald χ^2(2 d.f.) = 37.51, $p < 0.0001$), and also revealed a difference in the isoform INI (omnibus Wald χ^2(2 d.f.) = 7.98, $p = 0.019$). Pairwise comparisons revealed significant differences between C57BL/6J and both BALB/cJ and DBA/2J for C

Table 2
Percentages for observed *Htr2c* RNA isoforms

Isoform	BALB/cJ	C57BL/6J	DBA/2J
A	2.18	0.71	1.38
AB	11.26	6.83	15.23
ABC	2.46	6.51	5.16
ABCD	15.89	34.95	17.96
ABD	55.77	45.16	38.60
ABE	0.23	0.00	0.00
ABECD	2.37	0.37	1.06
AC	0.22	0.60	0.42
ACD	0.00	0.00	1.19
AD	2.59	0.97	0.00
AE	0.42	0.00	0.00
AEC	0.00	0.00	0.60
AECD	0.25	0.00	0.00
AED	0.25	0.00	0.00
B	0.25	0.00	2.42
BD	0.49	0.00	0.00
C	0.00	0.59	0.93
CD	0.22	0.00	1.11
D	0.65	1.93	4.05
E	0.23	0.00	0.00
ED	0.22	0.00	0.00
U	4.08	1.35	9.90

site editing and frequency of VSV, while differences between BALB/cJ and DBA/2J were not significant (see Table 4 and Fig. 2). With 95% confidence, the odds (# C sites edited/# C sites not edited) of a C site being edited in C57BL/6J is increased by a factor of 1.76–4.26 over that for BALB/cJ, and by a factor of 1.14–3.11 over that for DBA/2J. With 95% confidence, the odds (# VSV isoforms/# other isoforms) of an isoform being VSV in C57BL/6J is increased by a factor of 2.03–4.02 over that for BALB/cJ, and by a factor of 1.48–3.56 over that for DBA/2J. Pairwise comparisons for isoform INI showed that C57BL/6J differed significantly from DBA/2J, although the difference between DBA/2J and BALB/cJ borders significance (see Table 4 and Fig. 2). With 95% confidence, the odds of an isoform being INI in C57BL/6J versus DBA were decreased by a factor of 0.03–0.64.

Table 3
Percentages for predicted 5-HT_{2C} receptor isoforms

Isoform	BALB/cJ	C57BL/6J	DBA/2J
IDI	0.23	0.00	0.00
IDV	0.22	0.00	0.00
INI	4.08	1.35	9.90
INV	0.65	1.93	4.05
ISI	0.00	0.59	0.93
ISV	0.22	0.00	1.11
MNI	0.25	0.00	2.42
MNV	0.49	0.00	0.00
VDI	0.64	0.00	0.00
VDV	0.25	0.00	0.00
VGI	0.00	0.00	0.60
VGV	2.62	0.38	1.06
VNI	13.44	7.55	16.61
VNV	58.36	46.14	38.60
VSI	2.68	7.11	5.58
VSV	15.89	34.95	19.15

Table 4
GEE model pairwise strain comparisons for *Htr2c* RNA editing site C and isoforms INI, and VSV

DV	Comparison	OR[a]	95% CI	Probability
Site C	C = B[b]	2.77	1.76–4.36	0.000
	C = D	1.89	1.14–3.11	0.013
	B = D	0.68	0.42–1.11	0.126
INI	C = B	0.33	0.07–1.59	0.167
	C = D	0.14	0.03–0.64	0.012
	B = D	0.42	0.17–1.04	0.060
VSV	C = B	2.86	2.03–4.02	0.000
	C = D	2.30	1.48–3.56	0.000
	B = D	0.80	0.53–1.22	0.304

Abbreviations: C, C57BL/6J; B, BALB/cJ; D, DBA/2J; DV, dependent variable; OR, odds ratio; CI, confidence interval.
[a] See Bland and Altman (2000) for an explanation of odds ratios. For example, the OR of ∼2.8 for the difference in C site editing between C57BL/6J (43%) and BALB/cJ (21%) follows the percentages (changed to proportions): $(0.43/(1-0.43))/(0.21/(1-0.21)) = \sim 2.8$.
[b] Odds ratio denominator on the right.

Narrow sense heritability for variation in the amount of C site editing was 37%, using the method of Hegmann and Possidente (1981). Using $Omega^2$ as an estimate of total genetic determination, 42% of the variation in C site editing can be attributed to genetic effects ($F_{2,21} = 9.52$, $p = 0.001$). Narrow sense heritability for variation in the amount of isoform VSV was 42%, using the method of Hegmann and Possidente (1981). Using $Omega^2$ as an estimate of genetic determination, 48% of the variation in the amount of isoform VSV can be attributed to genetic effects ($F_{2,21} = 14.92$, $p < 0.0001$).

3.3. DNA sequencing

The genome of C57BL/6J has been sequenced (Waterston et al., 2002) and served as a reference sequence. Resequencing of C57BL/6J revealed complete conservation with the known genome sequence of *Htr2c*. Alignment of forward and reverse sequences for both BALB/cJ and DBA/2J, and 37 additional strains revealed complete conservation between these strains and C57BL/6J for the region of the RNA loop involved in editing of *Htr2c* (Fig. 3). Sequence variation in the *Htr2c* genomic region of RNA editing between BALB/cJ, C57BL/6J, DBA/2J and many other inbred strains cannot contribute to strain variation in *Htr2c* RNA editing profiles or to functional differences at the 5-HT_{2C} receptor.

3.4. 5-HT and 5-HIAA

While there were no significant differences between strains in 5-HT levels, there were strain differences in 5-HIAA (ANOVA $F_{2,26} = 7.79$, $p = 0.002$, omega squared = 0.32, effect size = 0.68). Fisher's protected LSD tests show C57BL/6J different from both BALB/cJ ($F_{1,26} = 5.34$, $p = 0.03$) and DBA/2J ($F_{1,26} = 15.37$, $p = 0.0006$); BALB/cJ and DBA/2J were not different ($F_{1,26} = 2.79$, $p = 0.11$). Serotonin turnover, indexed by the ratio 5-HIAA/5-HT, differed even more strongly

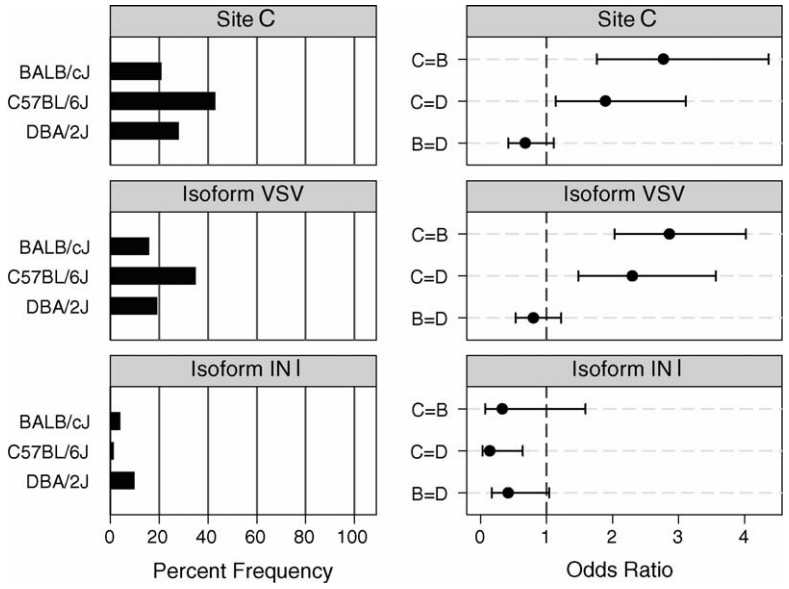

Fig. 2. *Htr2c* RNA editing differences in amygdala in BALB/cJ, C57BL/6J, and DBA/2J inbred mouse strains. The left panel displays the percentage of RNA editing at site C, of isoform VSV, and of isoform INI. The right panel displays the odds ratio and 95% CI for the pairwise null hypotheses for strain differences (C: C57BL/6J, B: BALB/cJ, D: DBA/2J; e.g., C = B is the null hypothesis for testing the difference between C57BL/6J and BALB/cJ, with BALB/cJ as the denominator in the odds ratio).

between strains (ANOVA $F_{2,26} = 12.39$, $p < .0001$, omega squared = 0.44, effect size = 0.89). Fisher's protected LSD tests show differences between all three strains, C57BL/6J and BALB/cJ ($F_{1,26} = 4.69$, $p = 0.04$), C57BL/6J and DBA/2J ($F_{1,26} = 24.70$, $p < 0.0001$), and BALB/cJ and DBA/2J ($F_{1,26} = 8.19$, $p = 0.008$) (Fig. 4, top panel). The ANOVA factor for strain explained more variability in 5-HIAA/5-HT than 5-HIAA, because of a significant positive correlation between 5-HT and 5-HIAA ($r = 0.39$, $p = 0.03$, $N = 29$, one DBA/2J sample missing) (Fig. 4, bottom panel). This indicates there is significant covariation between individual mice in 5-HT and 5-HIAA that is not explained by the strain differences in 5-HIAA.

4. Discussion

We discovered substantial relative reductions in C site editing and in the highly edited isoform VSV in the amygdala of BALB/cJ and DBA/2J mice compared to C57BL/6J mice. The unedited isoform INI was relatively decreased in C57BL/6J mice compared to DBA/2J mice. Strain C57BL/6J is thus characterized by having the lowest frequency of INI, an isoform with greater constitutive activity and potency, and the highest frequency of VSV, an isoform with significantly reduced constitutive activity and potency. Conversely, BALB/cJ and DBA/2J are characterized by having higher frequencies of INI and lower frequencies of VSV.

```
            Edit sites:  A B   EC    D
                         | |   ||    |
DBA/2J       1  GGTATGTAGCAATACGTAATCCTATTGAGCATAGCCGGTTCAATTCGCGGACTAAGGCCA   50
C57BL/6J     1  GGTATGTAGCAATACGTAATCCTATTGAGCATAGCCGGTTCAATTCGCGGACTAAGGCCA   50
BALB/cJ      1  GGTATGTAGCAATACGTAATCCTATTGAGCATAGCCGGTTCAATTCGCGGACTAAGGCCA   50

                              Exon/intron boundary:  *
                                                     |
DBA/2J      51  TCATGAAGATTGCCATCGTTTGGGCAATATCAATAGGTAATTATACCTGGCCATAGAATT  100
C57BL/6J    51  TCATGAAGATTGCCATCGTTTGGGCAATATCAATAGGTAATTATACCTGGCCATAGAATT  100
BALB/cJ     51  TCATGAAGATTGCCATCGTTTGGGCAATATCAATAGGTAATTATACCTGGCCATAGAATT  100

                                  D         CE   B  A
                                  |         ||   |  |
DBA/2J     101  GCAGCGGCTATGCTCAATACCTTCGGATTATGTACTGTGAACAACCTACAG           171
C57BL/6J   101  GCAGCGGCTATGCTCAATACCTTCGGATTATGTACTGTGAACAACCTACAG           171
BALB/cJ    101  GCAGCGGCTATGCTCAATACCTTCGGATTATGTACTGTGAACAACCTACAG           171
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Fig. 3. CLUSTAL alignment of sequenced mouse genomic *Htr2c* DNA for the putative dsRNA loop with edited exonic and complementary intronic regions highlighted. BALB/cJ, C57BL/6J, and DBA/2J are shown; 37 others strains that were also completely conserved are listed in Section 2.

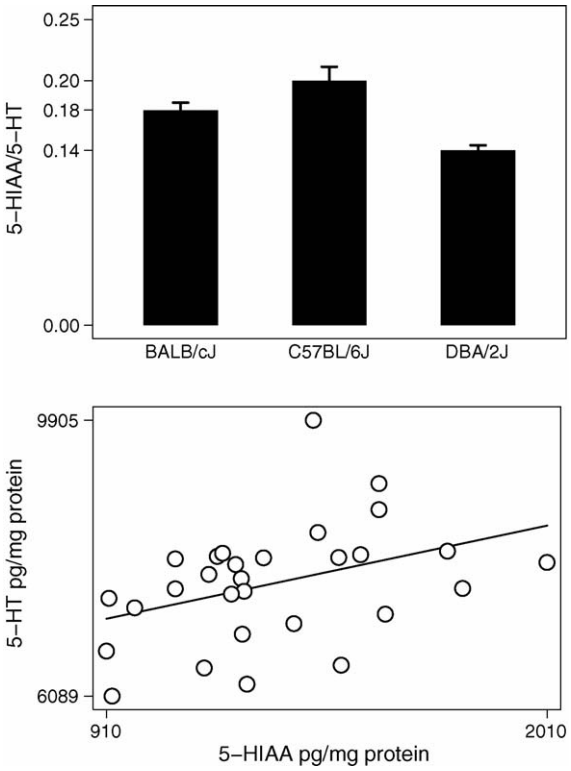

Fig. 4. Serotonin neurochemistry. The top panel displays a bar chart of strain means with standard errors of the ratio 5-HIAA/5-HT, an index of serotonin turnover. The bottom panel displays a scatterplot of 5-HT and 5-HIAA showing positive correlation: $r = 0.39$, $p = 0.03$.

Inbred mouse strain differences indicate a genetic basis for variation in RNA editing, and spur the use of quantitative genetic methods to investigate the causes and consequences (Phillips et al., 2002). The heritability estimates of variation in C site editing and frequency of isoform VSV (40–50%) are within the range typical of physiological or structural traits, and indicate strong genetic influence on standing differences in *Htr2c* RNA editing. The heritable differences in 5-HT_{2C} receptor RNA editing between C57BL/6J and DBA/2J inbred strains in particular suggest that the BXD recombinant inbred genetic reference panel of mice could be studied to locate influential genetic loci (QTL) and to correlate associated phenotypes (e.g. anxiety) (Peirce et al., 2004). This research possibility is further strengthened by the differences in anxiety behavior and serotonin turnover between C57BL/6J and DBA/2J inbred strains.

We confirmed, using a standard test of anxiety, the major corpus of behavioral literature describing BALB/cJ and DBA/2J as more anxious than C57BL/6J. We further discovered that, consistent with prior results implicating serotonin as a correlate of RNA editing (Gurevich et al., 2002b), both BALB/cJ and DBA/2J have decreased 5-HT turnover relative to C57BL/6J. Finally, we ruled out *Htr2c* sequence variation as an underlying cause of the strain differences in RNA editing profiles.

Studies of mouse neocortex *Htr2c* RNA editing from the Schmauss laboratory have also described inbred strain differences in basal profiles (Englander et al., 2005). The levels of INI and VSV in neocortex from C57BL/6J (~2% and ~25%, respectively) differed significantly from those in BALB/cJ (~70% and ~2%, respectively; taken from Fig. 1 of Englander et al., 2005). These estimates are strikingly different from those we describe here within the amygdala for these same strains (see Fig. 1 above). This suggests that strain and brain region interactions for isoform prevalence are to be expected, in addition to the strain and environmental challenge interactions observed by Englander et al. (2005). Exceptionally complex pictures may emerge from studies of *Htr2c* RNA editing in subdivisions of the cortex (such as the prefrontal cortex) that innervate subnuclei of the amygdala (Likhtik et al., 2005), suggesting that future studies should be multivariate by brain region or circuit.

The increased pool of reduced function isoforms in depressed suicide victims is consistent with the premise that the change in RNA editing profiles is a response to reduced synaptic serotonin function (Neumeister, 2003; Argyropoulos et al., 2004). Gurevich et al. (2002a,b) tested the effects of depletion and enhancement of 5-HT on *Htr2c* RNA editing. *Para*-chlorphenylalanine, which depletes 5-HT, increased the frequency of isoforms with higher sensitivity to serotonin (e.g. INI). Conversely, a serotonin agonist increased the frequency of isoforms that activate G-protein coupling less efficiently (e.g. VSV). Our data showing that serotonin turnover (5-HIAA/5-HT) is decreased within the amygdala of strains exhibiting decreased RNA editing is consistent with the suggestion of Gurevich et al. (2002a,b) that RNA editing of the 5-HT_{2C} receptor is regulated by synaptic levels of serotonin.

A functional SNP (Arg447Pro) in the tryptophan hydroxylase gene (*Tph2*) differs between DBA/2J or BALB/cJ (arginine at amino acid position 447) and C57BL/6J or 129 × 1/SvJ (proline at position 447) (Zhang et al., 2004). Serotonin levels and accumulation of the precursor 5-hydroxytryptophan, an index of serotonin synthesis rate, are reduced in the frontal cortex and striatum of BALB/cJ compared to 129/SvJ mice (Zhang et al., 2004). In a supplemental online figure, a significant difference between C57BL/6J and DBA/2J mice was found in striatal synthesis rate; serotonin levels were not reported. In amygdala, we found no differences in 5-HT levels between C57BL/6J and DBA/2J or BALB/cJ, although we did find large differences in serotonin turnover that align with the *Tph2* Arg447Prol SNP and are consistent with the reported differences in serotonin synthesis rate. Further study of the role of *Tph2* Arg447Pro SNP, serotonin, and its biosynthetic pathways, on *Htr2c* RNA editing profiles is suggested. Such studies are further motivated by recent work in humans on alterations in the functional activity of the amygdala related to *Tph2* and 5-HT transporter polymorphisms (Brown et al., 2005; Pezawas et al., 2005). Hypothetically, genetic polymorphisms in these key molecules may change synaptic levels of serotonin, which in turn alters RNA editing and functional properties of the 5-HT_{2C} receptor, leading to individual differences in functional imaging of the amygdala.

Two limitations temper the findings in this study. First, because a different cohort of animals was used for estimates of *Htr2c* RNA editing profiles, direct phenotypic correlation with

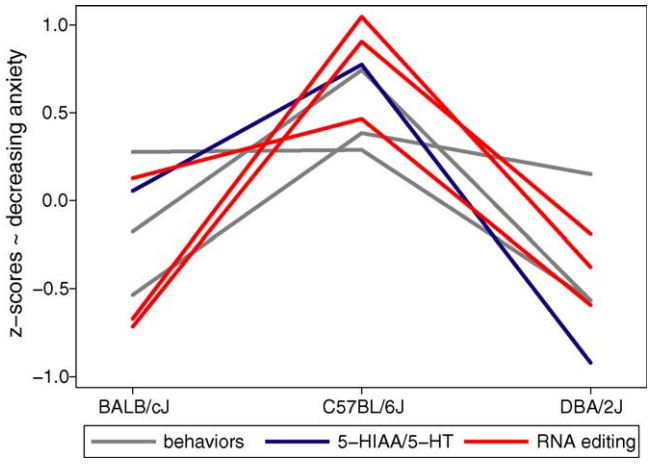

Fig. 5. A common pattern? Gray lines are the three anxiety measures reported for the light ↔ dark exploration. The blue line is serotonin turnover. Red lines are C site, INI, and VSV frequency. Each variable has been *z*-score transformed to plot all data on a common scale. INI and latency (light ↔ dark) measures have been inverted to be proportional to decreasing anxiety (up on the *y* axis). The apparent alignment represents an as yet untested and unproven hypothesis that serotonin levels influence *Htr2c* RNA editing levels thereby affecting amygdala activation and consequent anxiety levels.

serotonin levels or behavioral endpoints was not possible. By design, amygdala tissue that was used to isolate RNA could not also be used to quantify biogenic amines. Also, it is not known if exposure to the anxiety tests before RNA extraction might change the editing profiles. Second, because of the small number of strains used, genetic correlations (via strain means) could not be estimated. Although the data could well represent a spurious relationship, we found it striking that key *Htr2c* RNA editing traits, serotonin bioamine traits, and anxiety traits showed apparent alignment when plotted together (see Fig. 5). BALB/cJ and DBA/2J, compared to C57BL/6J, are characterized by a reduced pool of RNAs encoding receptor isoforms with decreased constitutive activity and decreased potency to stimulate intracellular signals. Future studies may confirm that differential titers of C site editing lead to differential activation of the amygdala and consequent anxiety differences. Based on what is known about activation of 5-HT_{2C} receptors in vivo in the amygdala, the lower titer of C site editing in BALB/cJ and DBA/2J predicts greater activation of amygdala.

Generating *Htr2c* editing profiles is laborious and expensive, requiring the sequencing of at least 30 RNA isolates per animal. By using a new, more rapid and less expensive method developed in our laboratory specifically for the purpose of quantifying *Htr2c* RNA editing profiles (Sodhi et al., 2005), future studies will be able to assess phenotypic and genetic correlations between anxiety or fear constructs and RNA editing profiles in a larger number of inbred strains and in more brains areas, such as the bed nucleus of the stria terminalis (Walker et al., 2003), nucleus accumbens (Sturm et al., 2003), ventral hippocampus (Alves et al., 2004), and prefrontal cortex (Iwamoto et al., 2005). Future studies will also assess whether pharmacological manipulations of anxiety alter the basal editing profiles reported here.

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