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Editing of the Serotonin 2C Receptor Pre-mRNA: Effects of the Morris Water Maze

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Abstract

The pre-mRNA encoding the serotonin 2C receptor, HTR2C (official mouse gene symbol, Htr2c), is subject to adenosine deamination that produces inosine at five sites within the coding region. Combinations of this site-specific A-to-I editing can produce 32 different mRNA sequences encoding 24 different protein isoforms with differing biochemical and pharmacological properties. Studies in humans have reported abnormalities in patterns of HTR2C editing in psychiatric disorders, and studies in rodents show altered patterns of editing in response to drug treatments and stressful situations. To further explore the biological significance of editing of the Htr2c mRNA and its regulation, we have examined patterns of Htr2c editing in C57BL/6J mice after exposure to the hidden platform version of the Morris Water Maze, a test of spatial learning that, in mice, is also associated with stress. In brains of both swimming controls and mice trained to find the platform, subtle time dependent changes in editing patterns are seen as soon as one hour after a probe trial and typically last less than 24 hours. Changes in whole brain with cerebellum removed differ from those seen in isolated hippocampus and cortex. Unexpectedly, in hippocampi from subsets of mice, abnormally low levels of editing were seen that were not correlated with behavior or with editing levels in cortex. These data implicate responses to spatial learning and stress, in addition to stochastic processes, in the generation of subtle changes in editing patterns of Htr2c.

Keywords

5HT2C receptor; inosine; protein isoforms; primer extension; spatial learning; stress

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1. Introduction

The serotonin 2C receptor, Htr2c, is unique among the serotonin receptors in that the pre-mRNA is subject to deamination of adenosine to inosine (Burns et al 1997). This editing occurs at five sites within a 15 nucleotide segment encoding the second intracellular loop (reviewed in Schmauss, 2002; Sanders-Bush et al 2003). Because inosine is interpreted by the ribosome as guanosine, editing alters individual codons, most often also altering the encoded amino acid. Figure 1a illustrates the repertoire of codon combinations that can result in a total of 24 different protein sequences. Within the protein sequence, the edited region starts two amino acids downstream from the conserved DRY motif that is believed to be involved in G-protein coupling (Ballesteros et al 1998; Moro et al 1993). Both mutation experiments and computational approaches predict that the sequence diversity created by editing will modulate protein function (Ballesteros et al 1998; Moro et al 1993; Visiers et al 2001), as originally suggested by Burns et al (1997). Indeed, in vitro studies have shown differences among several isoforms in biochemical and pharmacological properties that include affinity for serotonin, G-protein coupling, and responses to atypical antipsychotics (Niswender et al 1999; Herrick-Davis et al 1999; Fitzgerald et al 1999; Wang et al 2000; Price and Sanders-Bush 2000; Berg et al 2001; Niswender et al 2001; Price et al 2001; McGrew et al 2004; Marion et al 2004).

In vivo studies have shown that most of the 32 possible mRNA variants are produced in human and rodent brain (Burns et al 1997; Niswender et al 1999). In recent work, we have detected 28 of 32 mRNA variants in mouse brain, encoding 20 of 24 possible protein isoforms (Du et al 2006). However, there are both species-specific and brain region-specific differences in the proportions of the different variants, and only five or six variants typically are found at high frequency, with the remainder together accounting for only a few percent. Abnormalities in patterns of HTR2C editing have been reported in subsets of patients with schizophrenia and depression (Gurevich et al 2002a; Niswender et al 2001; Dracheva et al 2003), and baseline differences have also been reported among different inbred mouse strains (Englander et al 2005; Hackler et al 2006; Du et al 2006). Changes in editing patterns have been induced, in rodents, by exposure to tests of anxiety, including the forced swim test and learned helplessness, and by drugs, such as fluoxetine (Gurevich et al 2002b; Iwamoto et al 2005; Englander et al 2005; Yang et al 2004). Most of the changes in editing patterns induced by disease, genetic background, stress and pharmacological treatment are subtle and the corresponding diversity at the protein level has not yet been studied. Nevertheless, the cumulative data suggest that HTR2C pre-mRNA editing is biologically relevant.

Cavallaro et al (2002) classed the Htr2c as one of a number of “learning and memory genes” because transcript levels, as measured by microarray analysis, changed with time in rats exposed to the learning phase of the Morris Water Maze compared with those exposed to swimming alone. This observation prompts the question: do editing patterns change when transcript levels are dynamic and, if they do, how dynamic are the changes and are they relevant to learning?

Editing of HTR2C is effected by two adenosine deaminases that act on RNA, ADAR1 and ADAR2 (reviewed in Maas et al 2003). The ADAR2 gene (official gene name *ADARB1*) maps to human chromosome 21 and therefore is a candidate gene for the cognitive and behavioral features of Down syndrome (Villard et al 1997). If expression of ADAR2 is increased in Down syndrome, as predicted from gene dosage (trisomy of chromosome 21), it may alter patterns of HTR2C mRNA editing.

Based on these observation, we generated transgenic mice that carry an extra genomic copy of the human ADAR2 gene and examined editing of Htr2c at timed intervals after exposure to the Morris Water Maze. While results do not inform the role of ADAR2 in Down syndrome,

they illustrate dynamic patterns of editing that differ between brain regions, and that are associated with the stress of swimming and spatial learning. Results also suggest that stochastic processes contribute to editing patterns.

2. Methods

2.1 Mouse brain tissue

Transgenic mice, lines Tg(Adarb1)14Dn (Tg14Dn) and Tg(Adarb1)15Dn (Tg15Dn), carry the human BAC RP11-581A12 (BacPac Resources) which contains the entire ADAR2 gene. Both lines are in a C57BL/6J background and were produced by the transgenic facility at The Jackson Laboratory (Bar Harbor, ME). For the first set of experiments, using whole brain minus cerebellum, transgenic mice and wild type littermates were used. For the second set of experiments, using hippocampus and cortex, wild type C57BL/6J mice were purchased from The Jackson Laboratory. All mice were male and were housed at the Eleanor Roosevelt Institute for at least two months and until 6-8 months of age before testing in the Morris Water Maze (MWM). Mice from the same litter were kept in the same cage and were maintained on a 12:12 hour light/dark cycle (light on 7:00 AM) with unlimited access to food and water. Mice were euthanized by cervical dislocation at 1 PM and 7 PM, at timed intervals after the probe test of the MWM. Brains were dissected and immediately frozen in liquid nitrogen. All experimental methods involving mice were approved by the Animal Care and Use Committee of the University of Denver.

2.2 Expression of Adar2 in transgenic lines

Transcript levels and alternative splicing patterns of the endogenous mouse Adar2 and the transgene human ADAR2 were examined by standard semi-quantitative RT-PCR, using primers within exon 2 and exons adjacent to each alternatively spliced exon (Slavov and Gardiner, 2002). For Western analysis of total Adar2 protein (mouse plus human), an antibody recognizing the N-terminus and a custom antibody to a conserved peptide within the adenosine deaminase domain (Santa Cruz and Biosynthesis, respectively) were used against whole brain crude protein lysates. Editing levels of Htr2c and glutamate receptors, GluR2, 5 and 6, were examined by primer extension using published protocols (Niswender et al 2001; Chen et al 2000; Lai et al 1997).

2.3 Morris Water Maze

Mice were randomly assigned to one of three treatment groups: naïve (N), swimming controls (SC) or hidden platform trained (WM). To exclude possible differences caused by circadian rhythm, subsets of naïve mice were sacrificed at the same times as SC and WM mice.

The experimental apparatus was a circular pool (95 cm in diameter and 17 cm high) filled with water ($24^{\circ}\pm 0.5^{\circ}\text{C}$) made opaque with non-toxic white paint. For WM mice, a platform (6.5 cm diameter) was placed approximately 1 cm below the surface of the water and kept in the same position throughout the experiments. For SC mice, the platform was removed from the pool and “training” consisted of allowing them to swim for 60 seconds. The training protocol was similar to that of Cavallaro et al (2002). WM and SC mice received three blocks of training over two days. Each block consisted of four trials with a 15-20 minute rest period between trials. The starting position was randomized among the four quadrants, and each trial lasted 60 seconds (for SC mice) or until the mice located the platform (for WM mice). WM mice that did not find the platform within 60 seconds were guided to the platform. On day 1, SC and WM mice were trained in two blocks separated by a two-hour interval. On day 2, SC and WM mice received the third block of training, followed by a probe trial. During the probe trial, mice were given 60 seconds to search the pool with the platform removed. Performance of the mice was recorded (Videomex-V and Water Maze Program version 5, Columbus Instruments,

Columbus, OH), and the time to find the platform in each trial and the time spent in the training quadrant during the probe trial were analyzed. Table 1 lists the number of mice of each genotype, in each of the N, SC and WM groups, sacrificed at each time point.

2.4 Primer extension

In the first set of experiments, whole brains (left side) with cerebella removed were analyzed; in the second set of experiments, cortex and hippocampus were analyzed. RNA was extracted from individual brains and cortices using the standard guanidine-phenol method and from hippocampi, using Trizol (Invitrogen). One microgram of total RNA was used in first-strand cDNA synthesis with SuperScript reverse transcriptase (Invitrogen). The segment spanning the edited region was amplified (35–40 cycles) with primers 5' TGT GCT ATT TTC AAC TGC GTC CAT CAT G 3' and 5' CGG CGT AGG ACG TAG ATC GTT AAG 3', located in exon 5 and exon 6, respectively, of the mouse Htr2c mRNA (GenBank accession # NM_008312) using Taq polymerase (New England Biolab). Forty ng of PCR product were used as template for primer extension assays. Three primers (based on those of Niswender et al 2001 and Chen et al 2000) were used with different complements of ddNTPs, in a total of five reactions as shown in Figure 1a. The five adenosine residues that are targets of editing have been named, 5 to 3', as the A, B, E, C and D sites. Editing at the A site was measured in reaction 1 (Rxn 1) using forward strand primer extA-18mer, 5'CGCTGGACCGGTATGTAG3', with 4.3 mM ddTTP plus ddGTP, and 0.7 mM dATP and dCTP. Editing at the B site was measured in reaction 2 (Rxn 2) using forward strand primer extA-20mer, 5'CGCTGGACCGGTATGTAGCA3', with 6 mM ddATP and, in reaction 3 (Rxn3), with 6 mM ddGTP. Editing at the E, C and D sites was measured using reverse strand primer extD, 5'GAATTGAACCGGCTATGCTC3', in reaction 4 (Rxn 4), with 6 mM ddTTP and, in reaction 5 (Rxn 5), with 6 mM ddCTP. In reactions 2–5, non-chain terminator dNTPs were present at 1mM. Extension used standard protocols with Sequenase version 2.0 (USB); products were resolved on denaturing 15% polyacrylamide gels, exposed to a phosphor screen and scanned on a PhosphorImager (Molecular Dynamics, Storm 840). Signal intensities of bands and relative ratios were quantified using ImageQuant software (Molecular Dynamics).

The first set of calculations determined proportions of editing at individual sites (see band patterns in Figure 1b). Proportions of the A site editing were calculated from Rxn 1 as $A\% = A+ / (A+ + A-)$, where A+ and A- represent the intensities of corresponding bands in Figure 1b. In Rxn 2, levels of A+B+ (transcripts with the B site edited and the A site also edited) were measured as the ratio of the intensities of band A+B+E- over this plus A+B-. In Rxn 3, levels of A-B+ (levels of B site editing when the A site was unedited) were measured as the ratio of the intensities of band A-B+ relative to A-B+ plus A-B-. In Rxn 4, levels of E+C+D+ (levels of editing of the E site when the C and D sites are both edited) were measured as the ratio of the intensity of the band E+C+D+ over this plus E-C+D+. In Rxn 5, levels of E+C-D- (levels of E site editing when the C and D sites are both unedited) were measured as the ratio of the intensity of the band E+C-D- over this plus E-C-D-. Similarly, levels of C+D+ (levels of C site editing when the D site was edited) were measured in Rxn 4 as the ratio of the intensity of the band E+C+D+ plus band E-C+D+ over these plus band C-D+. Levels of C+D- (levels of C site editing when the D site was unedited) were measured in Rxn 5 as the ratio of the intensity of band C+D- over this plus the bands E-C-D- and E+C-D-. Levels of the D site editing were measured from Rxn 4, $D+T$, as the ratio of the intensity of the bands E+C+D+, E-C+D+ and C-D+ over these plus the band D- or from reaction 5, $D+C$, as the ratio of the intensity of the band D+ over this plus the bands C+D-, E+C-D- and E-C-D-. Note that, excluding experimental artifacts, values of $D+T$ and $D+C$ will be identical.

The second set of calculations determined proportions of transcripts encoding specific amino acids and amino acid combinations. As shown in Figure 1a, not all combinations can be

assayed. These calculations differ slightly from those described above for individual sites. Specifically, position 157 can code for isoleucine (unedited, A-B-), valine (A+B- and A+B+) or methionine (A-B+). Therefore, the proportion of transcripts encoding M was measured from Figure 1b, Rxn 3, as the ratio of the intensity of the band A-B+ (M) over this plus the bands A-B- (I) and A+ (V). Other combinations measured the proportions of G, S, N or D at position 159, in the context of either I or V at position 161 (Rxn 4 and Rxn 5). From Rxn 4, proportions of transcripts encoding SV were measured as the ratio of the intensity of the band E-C+D+ over this plus the bands E+C+D+, C-D+ and D-, and transcripts encoding NV+DV, were determined from the ratio of the intensity of the band C-D+ over this plus the bands E+C+D+, E-C+D+ and D-. From Rxn 5, proportions of transcripts encoding NI were measured as the ratio of the intensity of the band E-C-D- over this plus the bands E+C-D-, C+D- and D+, and proportions of SI+GI were measured as the ratio of the intensity of the band C+D- over this plus the bands E-C-D-, E+C-D- and D+. Note, due to limitations in the primer extension methodology, all determinations are approximate because of the existence of bands beyond those shown in Figure 1b that are too faint to measure.

2.5 Statistical analysis

All analyses used the GraphPad Prism 4 software. In the MWM, latency to find the hidden platform across three blocks of trials was compared with Two-Way Repeated Measures ANOVA, with genotype and block as independent variables. The percentage of search time in the training quadrant during the probe trial was compared with One-Way ANOVA, with genotype as the independent variable. In primer extension, the mean values from two to four replicate experiments were used, where the standard error of the mean (SEM) showed intra and inter-experimental variation of 1%-2%. Editing levels of individual sites and the levels of partial mRNA isoforms were compared with Two-Way ANOVA, with treatment (trained with hidden platform, swimming controls or naïve controls) and time past the probe trial (1, 6, 24 hours) as independent variables. Multiple comparisons were done by Bonferroni posttests.

2.6 Levels of Htr2c mRNA by Real Time PCR

Expression levels of the full length and the truncated splice variants of Htr2c mRNA (Xie et al 1996; Flomen et al 2004) were determined from whole brain (without cerebellum) by Real-Time PCR in two N, two SC and two WM mice at each time point. For full length HTR2C transcripts, primers mHT2CR F1 5'ATA GCC GGT TCA ATT CGC GGA CTA3' and mHT2CR R1 5'TGC TTT CGT CCC TCA GTC CAA TCA3' were used with the fluorescent probe mHT2CR L JOE-TCA TGA AGA TTG CCA TCG TTT GGG C-TAMRA. For the truncated HTR2C transcripts, primer mHT2CR Ftr 5'TGC TGA TAT GCT GGT GGG ACT ACT3' and mHT2C Rtr 5'AAC TGA AAC TCC GGT CCA GCG ATA3' were used with the probe tr JOE-TCA TGC CCC TGT CTC TGC TTG CAA-TAMRA. Real Time PCR primers and probes were designed by Primerquest (<http://scitools.idtdna.com/primerquest/>) and synthesized by IDT DNA, except for the GAPDH control (Applied Biosystems). Amplifications were performed on a ABI/PE5700 according to manufacturer's protocols. To construct the relative standard curve, each target (Htr2c-full length, Htr2c-truncated and GAPDH) was measured in a series of seven 1:2 dilutions of a control cDNA sample made from 10 microgram total RNA. Levels of full length or truncated variants of Htr2c were normalized to GAPDH, and then normalized to levels from naïve mice. Mean and standard deviations of transcript levels were calculated from three replicates. The expression level of Htr2c mRNA was expressed as an n-fold difference relative to naïve mice.

3. Results

3.1.1 Editing patterns in whole brain minus cerebellum

The first set of experiments was designed to assay the molecular and behavioral phenotypes of transgenic mice carrying a genomic copy of the human ADAR2 gene. For the molecular phenotype, two lines, Tg14Dn and Tg15Dn, were verified to express the human ADAR2 mRNA in addition to normal levels of mouse *Adar2* (Supplementary Figure 1). However, while levels and proportions of alternatively spliced variants of the mouse transcripts were normal, those for the human transcripts showed some abnormalities. In particular, inclusion of the Alu containing exon5a was increased to >95% in the transgenic brains, in contrast to approximately 50% seen in normal brain (Supplementary Figure 1). This is noteworthy because exon 5a transcripts result in a protein with a 50% reduction in deaminase activity. Western blots of whole brain lysates using two different antibodies, however, detected no increases (<15%) in *Adar2* protein levels. Lastly, no alterations in editing patterns of *Htr2c* mRNA or of the glutamate receptors (*GluR2*, 5 and 6) were detected in naive mice of either line (data not shown). Together, these data suggest that mechanisms for regulating *Adar2* activity compensate for gene dosage effects.

Because exposure to the MWM has been shown to alter *Htr2c* mRNA levels in rats (Cavallaro et al 2002), and because stimulation/stress might induce transgene-related phenotypes not apparent in naive mice, we next tested for possible transgene effects in mice exposed to swimming only and exposed to swimming plus learning the location of a hidden platform. As shown in Figure 2, there were no significant differences in performance between wild type and either line of transgenic mice. All were equally successful in learning the location of the platform, as measured by the decreased time to find the platform with increasing number of trials ($p < 0.01$, Two-Way Repeated Measure ANOVA, block) and by the amount of time spent searching the training quadrant when the platform was removed ($p = 0.84$, One Way ANOVA). The presence of the transgene, therefore, has no consequences for performance in the MWM.

Mice were euthanized at one, six and 24 hours after the final swimming session (probe test) and *Htr2c* editing patterns were assayed by primer extension as detailed in Figure 1a and represented in Figure 1b (adapted from Chen et al 2000; Niswender et al 2001). No differences in editing levels at any site or combination of sites were detected in transgenic mice of either line when compared to wild type controls, regardless of treatment or time (Supplementary Table 1). We next compared editing patterns between different treatment groups (N, SC and WM) and between time points (1, 6 and 24 hours). Based on the observation of no *Adar2* protein increases, no abnormalities in editing patterns, and no deficit in the MWM, we concluded that the presence of the transgene does not affect the parameters of interest here. Accordingly, data for the three genotypes (wild type, tg14, tg15) were pooled to improve statistical significance for comparative analysis. Table 2 summarizes the significant similarities and differences. Note that there is little variation in measured values between replicates and between mice of the same treatment, as reflected in the typical SEM of 1%-2% and as shown in Supplementary Table 1.

As shown in Table 2, editing levels at the A site did not change with either treatment or time ($F_{2, 45} = 2.03$, $p = 0.14$). However, levels of A+B+, that averaged 75% in N mice, were significantly increased in both SC and WM mice at one hour, where they averaged 81%, remaining high in WM mice at six hours (82%; $F_{2, 45} = 20.29$, $p < 0.0001$). In contrast, no differences in the levels of A-B+ were detected in any comparison ($F_{2, 45} = 1.18$, $p = 0.32$).

Analysis of editing at the E and C sites is complicated in that the method used here couples editing at the E and C sites to the editing status at the D site. To distinguish measurements with and without editing at the D site, levels of the E and C site editing are expressed in two columns

(Table 2; ddT, Rxn 4; ddC, Rxn 5). Editing of the E site is measured when the C and D sites are both edited (E+C+D+), or when the C and D sites are both unedited (E+C-D-). Similarly, editing of the C site is measured when the D site is edited (C+D+ or unedited (C+D-).

Levels of E+C+D+ were significantly affected by treatment ($F_{2,44} = 9.42$, $p < 0.001$). In naïve mice, the level of E+C+D+ averaged 5%. While this was not significantly different in SC mice (averaging 7%), it was significantly increased in WM mice at one hour, where it averaged 9% ($p < 0.01$). Levels of E+C-D- were significantly affected by time ($F_{2,45} = 4.70$, $p < 0.05$), however, significant interactions between treatment and time were also observed ($F_{4,45} = 4.09$, $p < 0.01$), indicating that treatment had different effects across the three time points. In naïve mice, levels of E+C-D- averaged 10%. This was significantly increased, to 19%, in SC mice at six hours ($p < 0.01$) which was also significantly higher than in WM mice, at 12%, at six hours ($p < 0.05$).

Levels of C+D+ also were significantly affected by time ($F_{2,45} = 3.26$, $p < 0.05$), while levels of C+D- did not change with either time or treatment ($F_{2,45} = 2.00$, $p = 0.15$, treatment; $F_{2,45} = 2.14$, $p = 0.13$, time).

Levels of editing at the D site changed with treatment (in ddT reactions, $F_{2,45} = 8.99$, $p < 0.001$; in ddC reactions, $F_{2,45} = 5.07$, $p < 0.05$). In naïve mice, they averaged 86%, but, in WM mice, they decreased to 82% and 80% at one and six hours ($p < 0.05$ and $p < 0.01$, respectively).

To determine if Htr2c editing patterns were altered at least in part because of changes in levels of Htr2c mRNA, Real Time PCR was used to quantitate amounts of the full length and truncated splice variants of Htr2c. There were no significant differences between naïve mice and SC or WM mice, although there was a trend towards a decrease with swimming (Table 3).

3.1.2 Alterations in Htr2c mRNA isoforms in whole brain minus cerebellum

In order to deduce the consequences for protein isoforms, mRNA sequences were analyzed in two segments, the segment containing the A and B sites and the segment containing the E, C and D sites. Editing at the A site, with or without editing at the B site, results in valine, and therefore the frequency of mRNA variants encoding valine at position 157 will be reflected in the level of editing at the A site, regardless of editing at the B site (Rxn 1, Figure 1). Consistent with editing at the A site, the frequency of mRNA isoforms encoding valine at position 157 did not change with treatment or time. Similarly, levels of transcripts encoding isoleucine (A-B-) and methionine (A-B+) were unchanged ($p = 0.71$ and 0.37 , treatment and time; $p = 0.24$ and 0.77 , treatment and time, respectively).

Editing at the E, C and D sites creates eight amino acid combinations at positions 159 and 161. As shown in Figure 1a, the proportions of transcripts encoding GV, SV and NV+DV and NI, DI and SI+GI can be calculated. Significant changes are shown in Table 4. Treatment had an overall significant effect on the proportion of transcripts encoding NI ($F_{2,45} = 3.82$, $p < 0.05$). In contrast, the proportion of transcripts encoding SV did not change with either treatment or time ($F_{2,45} = 1.57$, $p = 0.22$, treatment; $F_{2,45} = 2.29$, $p = 0.11$, time). The proportions of transcripts encoding NV+DV and SI+GI both changed with treatment ($F_{2,45} = 3.85$, $p < 0.05$; $F_{2,45} = 11.62$, $p < 0.0001$). In WM mice at one hour, levels of NV+DV (65% in N mice) decreased significantly to 58% ($p < 0.05$), and levels of SI+GI (4% in N mice) increased significantly to 8% ($p < 0.01$). The latter remained high at 6 hours, where they averaged 7% ($p < 0.05$).

3.2.1 Editing in hippocampus and cortex

The significant changes in editing patterns seen in whole brain minus cerebellum are assumed to be a summation of changes that may vary among brain regions. Because the Morris Water Maze tests spatial learning and because swimming induces stress in mice, we next assayed

editing in hippocampus and cortex from a second set of mice composed entirely of wild type C57BL/6J mice. This set included 13 naïve mice (3, 5 and 5 sacrificed at one, six and 24 hours) and three SC and WM mice sacrificed at each time point. Results for individual mice are presented in Supplemental Tables 2 and 3. Examination of the data for hippocampus unexpectedly showed that there were significant differences among individual mice, unrelated to treatment or time. Specifically, mice can be divided into two groups: (1) the majority, 25 of the total of 31 exhibited high levels of editing at the A site, averaging 78%, similar to those seen in whole brain minus cerebellum of naïve mice (Table 1), and (2) the remaining 6 mice showed significantly lower levels of editing at the A site, five averaging ~25%, and one at 55% (Figure 3a,b). As shown in Table 5, editing levels at additional sites, most often the B site, were also affected. This variation cannot be attributed to differences in the time of day at which the mice were sacrificed (note that while all three N mice at one hour showed low levels, five N mice at 24 hours, the same time of day, did not), the order in which the mice were euthanized at any time point (which might affect stress levels), or to quality of the isolated RNA or RT-PCR product. Because the mice are genetically identical, differences due to polymorphisms in either the Htr2c gene or the adenosine deaminases that carry out editing can be ruled out. These points suggest that stochastic processes play a role, a conclusion that is further supported by the observation that editing levels in the cortex of the same mice do not show these patterns of individual variation (Figure 3c,d), with the exception of the N1-3 mouse at the A site (55%). In addition, of the 42 mice analyzed in whole brain in the first experiments, only one showed a moderately low level of editing at the A site (54%).

The appearance of mice with low and high editing levels complicates the comparisons between naïve mice, and SC and WM mice and could obscure time/treatment dependent differences. Therefore, we restricted analysis in hippocampus to only those mice with the (predominant) high levels of editing at the A site, deleting the five mice with A site editing <30%.

3.2.2. Editing in hippocampus

Significant changes in hippocampus of SC and WM mice are shown in Figures 4 (additional data are provided in Supplementary Tables 2, 4 and 5). In hippocampus, levels of editing at the A site did not change with either treatment or time (treatment, $F_{2,39} = 0.33$, $p=0.72$; time, $F_{2,39} = 0.79$, $p=0.46$) and nor did those of A+B+ (treatment, $F_{2,39} = 1.24$, $p=0.30$; time, $F_{2,39} = 0.62$, $p=0.54$). However, levels of A-B+ significantly changed with treatment ($F_{2,39} = 5.45$, $p<0.01$) (Figure 4a). In naïve mice, levels of A-B+ averaged 31%, which decreased in SC and WM mice, at one, six and 24 hours, to 23%, 21% and 23% and 17%, 18% and 21%, respectively.

Levels of E+C+D+ were significantly affected by both treatment and time (treatment, $F_{2,39} = 4.51$, $p<0.05$; time, $F_{2,39} = 5.52$, $p<0.01$) (Figure 4b). In naïve mice, levels averaged 9%, and were decreased to 5% in both SC and WM mice at 6 hours ($p<0.05$). In contrast, levels of E+C-D- did not change with either treatment or time (treatment, $F_{2,39} = 0.03$, $p=0.97$; time, $F_{2,39} = 0.41$, $p=0.67$).

Levels of C+D+ were significantly affected by time ($F_{2,39} = 5.00$, $p<0.05$) (Figure 4c), while levels of C+D- and levels of the D+ did not change with either time or treatment.

Because of the significant effect of treatment on levels of A-B+, levels of transcripts encoding M at the position 157, compared to those encoding I or V, changed with treatment ($F_{2,39} = 3.70$, $p<0.05$) (Figure 4d). Although it did not reach significance, levels of transcripts encoding methionine were decreased in SC and WM mice (4%-5%) compared to naïve mice (7%).

The proportion of transcripts encoding SV (E-C+D+) changed with time (time, $F_{2,39} = 3.57$, $p < 0.05$) (Figure 4e), however, the proportion of transcripts encoding NI (E-C-D-), NV+DV (C-D+) or SI+GI (C+D-) did not change with either treatment or time.

3.2.3. Editing in cortex

Site-specific editing and amino acid combinations with significant changes in cortex are shown in Figure 5 (see also Supplementary Tables 3, 6 and 7). Levels of A-B+ changed significantly with treatment ($F_{2,48} = 7.24$, $p < 0.01$), and a showed significant interaction between treatment and time (treatment has different effects at each time point) ($F_{4,48} = 2.88$, $p < 0.05$) (Figure 5a). Relative to 35% in naïve mice, levels of A-B+ were increased, to 54%, in SC mice at 1 hour ($p < 0.05$) and to 46%, in WM mice at 1 hour.

Levels of E+C+D+ were significantly affected by time ($F_{2,48} = 3.66$, $p < 0.05$) (Figure 5b), increasing in SC and WM mice, particularly at 6 hours. Treatment had a significant effect on levels of D_T ($F_{2,48} = 3.70$, $p < 0.05$) (Figure 5c). (Note that D_C values were not analyzed; see Supplementary Table 6.)

Time had significant effects on the proportion of transcripts encoding methionine (A-B+) compared to those encoding valine and isoleucine ($F_{2,48} = 4.38$, $p < 0.05$) (Figure 5d), increasing in SC and WM mice at 1 hour (14% and 9%, respectively), compared to naïve mice at 7%. The proportions of transcripts encoding SV, NI, NV+DV or SI+GI did not change with either treatment or time.

4. Discussion

The MWM was chosen as a behavioral task because previous work by Cavallaro et al (2002) showed that it causes changes, that differ between the SC and WM mice, in the level of Htr2c mRNA during the 24 hours after the last exposure to swimming. Those experiments measured Htr2c mRNA levels in rat hippocampus by microarrays, and while not dramatic (the greatest was a four fold decrease), changes were significant. Such changes were not replicated here in mice by quantitative RT-PCR, but there are reasonable explanations. First, the protocol for the MWM used here differed slightly from that of Cavallaro et al (2002), specifically in that we included a probe trial for all mice, after the three blocks of training. Thus, the time of euthanasia was delayed until after an additional swimming episode beyond the learning phase. By this time point in Cavallaro et al (2002), rat hippocampal levels differed from naïve controls by only 50% which is at the limit of our detection. A similar decrease is suggested, however, by data in Table 3 showing a trend towards significant decreases. Regardless, the probe trial was necessary here because the learning phenotype of the ADAR2 transgenic mice was unknown. Second, while Cavallaro et al (2002) examined mRNA levels in hippocampus, our measurements were made in whole brain (with cerebellum removed) which could obscure region-specific changes.

The hidden version of the MWM is an attractive behavioral test because it couples spatial learning (mice are required to use spatial cues from the environment to learn the location of the platform) which is considered to be a hippocampal measure, with a stressful situation (because the mice find water aversive) (Schimanski and Nguyen 2004). For Down syndrome-related studies, it has the added attraction of being the most commonly used test, providing a robust phenotype in many mouse models (Escorihuela et al 1998; Sago et al 1998; Chabert et al 2004; Martinez-Cue et al 2005; Ahn et al 2006). There are, however, significant drawbacks to using the MWM when assaying a molecular phenotype. The procedure is extended over multiple days and multiple trials. The time frame in which molecular changes occur that are indicative of learning is not known, and such critical time points are difficult to predict and

will require rather large numbers of mice to ascertain. Nevertheless, in spite of the challenges, several useful observations were made.

The presence of increased levels of ADAR2 mRNA in transgenic lines did not lead to corresponding increased protein levels. This could be due to the heterologous system of expressing the human gene in the mouse background. It could also be due to normal regulatory mechanisms. Although we did not observe any increase in levels of transcripts encoding the truncated ADAR2 open reading frame that is produced by self-editing (this transcript encodes a non-functional protein that regulates ADAR2 protein levels in a negative feedback mechanism (Rueter et al 1999; Feng et al 2006)), we did see an increase in transcripts that contain exon 5a, which would result in a less active protein. Our results are not inconsistent with the (limited) studies of samples (two) from patients with Down syndrome, where no increases in editing levels of glutamate receptors were seen (Kawahara et al 2004). Nevertheless, our observations imply that regulatory mechanisms compensate for dosage of the ADAR2 gene. While not a unique observation, this is not commonly seen with many trisomic genes in Down syndrome and segmental trisomy mouse models, where 50% increases in protein levels have been observed (Gardiner 2003). While we cannot rule out other effects of the transgene, perhaps with brain region or cell type specificities, at the level of resolution of our analysis, transgene effects are not observed in editing patterns or in MWM performance. It is therefore reasonable to pool data from the three genotypes for analysis of treatment induced editing changes.

In hippocampus, cortex and whole brain (wild type and transgenic mice, without cerebellum), changes in editing patterns were observed as soon as one hour after the probe trial or the last swimming episode. These changes are modest, but the low inter-individual variation in measurements leads to differences that are statistically significant between treatment groups. Changes were most complex in whole brain, where patterns associated with B, E, C and D editing were affected. Of these, several were specific to WM mice (Table 2), suggesting effects of learning versus effects of swimming. In hippocampus and cortex, editing levels in naive mice were similar to each other, and differed from those in whole brain only in levels of A-B + (>30% compared to 20%, respectively). With learning and swimming, while both hippocampus and cortex showed changes, none discriminated between SC and WM mice, with the exception of A-B+ in cortex from SC mice at one hour. Levels of E+C+D+ were affected in both hippocampus and cortex, while changes in C+D+ were specific to hippocampus and changes in D+ were specific to cortex. That the patterns in hippocampus and cortex do not account for the observations in whole brain suggest that additional brain regions contribute to the dynamic responses of editing with exposure to the MWM.

Changes in editing patterns in all brain regions were short lived, largely returning to normal by six hours. This suggests that previous studies of C57BL/6J exposed to the Forced Swim test (Englander et al 2005) may have failed to detect significant changes in editing patterns because the assays were carried out 24 hours after swimming. Certainly C57BL/6J differed from BALB/c, which showed substantial changes, however, time frame, as well as patterns, may need to be considered.

Not all significant changes alter the protein coding sequence, and it is the predicted amino acid changes (summarized in Table 6) that dictate the possible functional consequences. The most consistent changes following MWM exposure involve amino acid position 159 (SI, GI, etc) and levels of M. This is consistent with observations of Wang et al (2000) who postulated a dominant role for position 159 (158 in the human sequence) in fine tuning receptor function. In hippocampus and cortex, levels of M changed in opposite directions.

Lastly, subsets of wild type C57BL/6J mice showed anomalously low levels of editing in hippocampus. Observed predominantly at the A and B sites, this implicates ADAR1 (Hartner et al 2004; Wang et al 2004), as opposed to ADAR2 which preferentially edits the C and D sites (Higuchi et al 2000). ADAR1 also is functionally distinct from ADAR2 in that ADAR1 shuttles between the nucleus and the cytoplasm, while ADAR2, consistent with the role in pre-mRNA editing, is restricted to the nucleus. Elimination of contributions from various experimental factors, such as circadian effects or RNA quality, and the observation of normal levels of editing in the cortex of the same mice suggests that stochastic processes may contribute to editing patterns. This is perhaps not surprising, given the observations of inter-individual variation in other molecular parameters (e.g. mRNA levels among inbred rats (Alfonso et al 2002)). Low editing is, however, relatively rare, because of the 42 mice analyzed in the whole brain experiments, only a single animal showed low levels (Table 3). If indeed due to stochastic events, such variation will further complicate human studies where genetic backgrounds are not uniform. It is noteworthy that, although numbers of mice are small, there is no indication that low levels of editing affect performance in the MWM. Ultimately, to ascertain the biological significance of Htr2c editing, with respect to both variation in basal patterns and responses to environmental influences, it remains important to extend observations to the protein level and to expand information on the functional properties of the Htr2c isoforms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Htr2c, serotonin receptor 2C; I, inosine; A, adenosine; G, guanosine; MWM, Morris Water Maze; SC, swimming control.

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a)

Figure 1.

Primer extension analysis. a) Schematic of primer extension assays. The genomic sequence spanning the edited region is shown in the center, 5' to 3'. The five A residues that can be edited are named A-E in the order indicated. Primer extension reactions 1-5 are shown with the corresponding dideoxynucleotide chain terminator. Horizontal lines indicate positions at which chain termination may occur. Products obtained in each reaction are named for the edited site combination and the corresponding encoded amino acids (underlined). + indicates the site is edited to inosine; - indicates it remains the genomic adenosine. b) Representative gels for reactions 1-5. Bands are labeled by edited status (site + or - editing) and length in nucleotides. P, primer.

Figure 2.

Results of Morris Water Maze with wild type (WT) C57BL/6J and two lines of ADAR2 transgenic mice, Tg14 and Tg15. a) Latency: learning in the MWM is indicated by the decreased amount of time (in seconds) required to find the hidden platform with increasing number of training sessions. Both wild type and transgenic mice learned to locate the hidden platform in approximately 30 seconds after three blocks of training. b) Results of the probe trial (hidden platform removed) of Morris Water Maze test. After two days of training in the MWM, mice were tested immediately in a single probe trial. Learning is assessed by recording the percentage of time spent in the training quadrant (former location of the platform). There

is no significant difference between wild type and transgenic mice in preference for the training quadrant ($p=0.84$, One Way ANOVA). TQ, training quadrant.

Figure 3.

A subset of mice show low levels of editing at the A and B sites in hippocampus. a) Proportion of transcripts edited at the A site in 13 naive (N), 9 swimming control (SC) and 9 mice trained to find the platform (WM). Five mice are edited at <30% while 25 are edited at $\geq 70\%$. b) Proportion of transcripts edited at both the A and B sites in the same animals. c) Animals with low levels of editing at the A site in hippocampus (Hp) show high levels of editing at the A site in cortex (Cr). d) Animals with low levels of A+B+ editing in hippocampus show high levels of A+B+ in cortex.

Figure 4.

Significant changes in editing patterns in hippocampus. a) Levels of A-B+ were affected by treatment ($p < 0.01$). b) Levels of E+C+D+ were affected by treatment ($p < 0.05$) and time ($p < 0.01$). c) Levels of C+D+ were affected by time ($p < 0.05$). d) Levels of transcripts encoding methionine (M) were affected by treatment ($p < 0.05$). e) Levels of transcripts encoding serine-valine (SV) were affected by time ($p < 0.05$).

Figure 5.

Significant changes in editing patterns in cortex. a) Levels of A-B+ were affected by time ($p<0.01$); time and treatment show an interaction ($p<0.05$). b) Levels of E+C+D+ are affected by time ($p<0.05$). c) Levels of D+ were affected by treatment ($p<0.05$). d) Levels of transcripts encoding methionine were affected by time ($p<0.05$).

Table 1

Numbers of mice by genotype, treatment and time of sacrifice.

	WT ^a	Tg14	Tg15	WT ^b
N	6	*	*	13
SC1	3	2	1	3
WM1	5	4	2	3
SC6	2		2	3
WM6	2	1	3	3
SC24	2		2	3
WM24	2		3	3

^a control C57BL/6J used in experiments with whole brain minus cerebellum.

^b control C57BL/6J used in experiments with cortex and hippocampus. N, naive mice. SC1,6, 24: swimming controls sacrificed at 1, 6 and 24 hours after the probe test. WM1, 6, 24: water maze trained mice sacrificed at 1, 6 and 24 hours after the probe test.

* naive mice tested previously.

Table 2
Editing levels of individual sites in brains minus cerebellum.

Hr	Tr	#	A+	A+B+	A-B+	E+C+D+	E+C-D-	C+D+	C+D-	D _T	D _C
			R1	ddT ^a	ddC	ddT ^b	ddC ^c	ddT ^d	ddC	ddT ^e	ddT ^f
	N	6	74% ±1	75% ±1	20% ±2	5% ±0.3	10%±1	25% ±2	32%± 3	86% ±2	88%± 1
1	SC	6	78% ±1	81% ^{***} ±1	26% ±3	7% ±1	9%±1	29% ±1	32%± 2	83% ±1	83%± 2
	WM	11	73% ±2	81% ^{***} ±1	20% ±3	9% ±1 ^{**}	13%±2	29% ±2	34%± 2	82% [*] ±1	75%± 5
6	SC	4	75% ±2	78% ±1	22% ±2	6% ±1	19%±3 ^{**}	26% ±1	40% ±2	83% ±2	84%± 2
	WM	6	77% ±1	82% ^{***} ±1	23% ±1	6% ±1	12%±1	23% ±0.3	37%± 2	80% ^{***} ±1	81%± 2
24	SC	4	77% ±1	77% ±1	24% ±3	6% ±1	10%±1	26% ±1	36%± 2	82% ±1	85%± 1
	WM	5	79% ±1	77% ±1	22% ±4	8% ±2	8%±1	26% ±1	29%± 3	84% ±1	73%± 9

Hr, hours after the last exposure to swimming. Tr, treatment, N, naive; SC, swimming control; MW, hidden platform trained. #, number of mice analyzed in each group. Data are means±S.E.M of each group. Shaded column headings indicate significant differences with treatment or time. Shaded individual time/treatment data indicate significant differences in pairwise comparisons. Results analyzed by Two-Way ANOVA: treatment had a significant effect,

^a p<0.0001,

^b p<0.05.

^c time had a significant effect, p<0.05 and time and treatment showed a significant interaction, p<0.01. Time had a significant effect,

^d p<0.05. Treatment had a significant effect,

^e p<0.001,

^f p<0.05. Pairwise comparisons performed by Bonferroni posttest:

* indicate p<0.05,

** p<0.01 and

*** p<0.001 relative to naïve controls.

Table 3

Expression of 5HT2CR splice variants in whole brain minus cerebellum.

Hr	Tr	Full Length ^a	Truncated ^b
1	N	1.0±0.14	0.91±0.14
		0.96±0.22	1.00±0.18
	SC	0.71±0.05	0.84±0.18
		0.82±0.13	0.79±0.13
	WM	0.51±0.08	0.82±0.19
6	SC	0.5±0.14	0.86±0.15
		0.76±0.14	0.77±0.12
	SC	0.65±0.14	0.87±0.10
		0.46±0.06	0.83±0.14
	WM	0.67±0.18	0.79±0.16
24	SC	0.73±0.20	1.11±0.16
		1.03±0.26	1.01±0.15
	WM	0.52±0.05	0.84±0.06
		0.67±0.14	0.61±0.11

^a ratio of full length 5HT2CR mRNA normalized to GAPDH.^b ratio of the truncated variant normalized to GAPDH. Data are means ± S.D. of three replicates of real time PCR reactions from one cDNA dilution, for each of two individuals from the N, SC and WM groups examined at each time point. Abbreviations are as Table 1.

Table 4
Levels of partial mRNA isoforms in whole brains minus cerebellum.

Hr	Tr	#	A-B+ M	E-C+D+ SV	E-C-D- NI ^a	C-D+ NV+DV ^b	C+D- SH+GI ^c
1	N	6	4%±0.2	21%±1	8%±1	65%±3	4%±1
	SC	6	5%±1	22%±1	11%±2	59%±1	6%±0.4
	WM	11	5%±1	20%±1	16%±4	58%±1 [*]	8%±1 ^{**}
6	SC	4	4%±0	20%±1	8%±0.4	62%±2	7%±1
	WM	6	5%±0.2	17%±0.4	11%±1	62%±1	7%±1 [*]
24	SC	4	5%±1	20%±1	10%±0.3	62%±1	6%±1
	WM	5	5%±2	20%±1	19%±9	63%±1	7%±2

See legend to Table 2. Treatment had a significant effect,

^a p<0.05;
^b p<0.05;
^c p<0.0001.
^{*} p<0.05,
^{**} p<0.01 relative to naïve controls.

Table 5

Site-specific low editing levels in a subset of mice.

	A	A+B+	A-B+	E+C+D+	E+C-D-	C+D+	C+D-	D+
N-Br^d	74	75	20	5	10	25	32	86
WM1-11	54	81	29	38	7	42	20	82
N-Hp^d	78	80	33	9	14	27	36	84
N1-1	23	52	7	3	14	23	23	78
N1-2	26	59	8	3	18	22	22	78
N1-3	55	76	13	8	19	29	43	84
SC24-1	24	55	8	6	12	23	27	78
WM6-3	29	56	11	6	7	23	23	81
WM24-3	33	67	7	7	7	24	23	82
N-Cx^d	83	75	34	7	12	24	37	82
N1-3	55	65	39	6	17	29	39	82

^d average editing levels at each site and combination in N, naive mice, in Br, brain, Hp, hippocampus and Cx, cortex. Numbers after N, SC and WM indicate time of sacrifice-individual in the group. Low levels are shaded.

Table 6

Summary of protein isoform changes

Region	Tr ^a	Hr ^b	AA	change
Whole brain	WM	1	NV+DV	65% to 58%
	WM	1	SI+GI	4% to 8%
	WM	6	SI+GI	4% to 7%
	SC,WM	1-24	NI	increase
Hippocampus	SC<WM	1-24	M	decrease
	SC,WM	1-24	SV	increase
Cortex	SC	1	M	increase
	WM	6	M	decrease

^a treatment;^b time of sacrifice after probe test;^c significant amino acid change