The roles of phospholipase C activation and alternative ADAR1 and ADAR2 pre-mRNA splicing in modulating serotonin 2C-receptor editing in vivo

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ABSTRACT

The serotonin 2C receptor (5-HT_{2C}), a Gq-protein-coupled neurotransmitter receptor, exists in multiple isoforms that result from RNA editing of five exonic adenosines that are converted to inosines. In the adult brain, editing of 5-HT_{2C} pre-mRNA exhibits remarkable plasticity in response to environmental and neurochemical stimuli. Here, we investigated two potential mechanisms underlying these plastic changes in adult 5-HT_{2C} editing phenotypes in vivo: activation of phospholipase C (PLC) and alternative splicing of pre-mRNA encoding the editing enzymes ADAR1 and ADAR2. Studies on two inbred strains of mice (C57Bl/6 and Balb/c) revealed that sustained stimulation of PLC—a downstream effector of activated Gαq protein—increased editing of forebrain neocortical 5-HT_{2C} pre-mRNA at two sites known to be targeted by ADAR2. Moreover, changes in relative expression of the alternatively spliced “a” and “b” mRNA isoforms of ADAR1 and ADAR2 also correlate with changes in 5-HT_{2C} editing. The site-specific changes in 5-HT_{2C} editing detected in mice with different “a” over “b” ADAR mRNA isoform ratios only partially overlap with those evoked by sustained PLC activation and are best explained by the increased editing efficiency of ADAR1. Thus, activation of PLC and alternative splicing of ADAR pre-mRNA have both overlapping and specific roles in modulating 5-HT_{2C} editing phenotypes.

Keywords: serotonin 2C receptor; RNA editing in vivo; phospholipase C; ADAR1; ADAR2; alternative splicing

INTRODUCTION

The conversion of adenosine (A) to inosine (I) by RNA editing is a widespread post-transcriptional mechanism to generate diversity among RNA transcripts (Bass 2002). Many A-to-I editing sites are predicted to exist in noncoding regions, including Alu repeats and the 5′ and 3′ untranslated regions of RNA (Levanon et al. 2004; Li et al. 2009). Yet, only a few A-to-I editing sites were found in coding regions of neuronal genes; included among these are five exonic adenosines in pre-mRNA encoding the 5-HT_{2C}, named the A-, B-, C′-, C-, and D-editing sites (Bass 2002; Werry et al. 2008). From all known neuronal A-to-I editing substrates, the five exonic adenosines in the 5-HT_{2C} pre-mRNA exhibit the greatest in vivo editing plasticity. Editing of these five adenosines is modulated by sustained changes in

serotonergic neurotransmission (Gurevich et al. 2002), and editing is increased after adult (Englander et al. 2005) and early life stress (Bhansali et al. 2007).

The molecular mechanisms underlying the plastic changes in 5-HT_{2C} pre-mRNA editing brought on by these environmental stimuli remain elusive. One potential mechanism underlying changes in editing efficiency could involve the cytosolic signaling cascade triggered by the G-protein subunit Gαq, which can be activated by 5-HT_{2C}-receptor stimulation. This is supported by findings that sustained activation of 5-HT_{2C} in vivo leads to increased editing of 5-HT_{2C} pre-mRNA (Gurevich et al. 2002), and that increased 5-HT_{2C} pre-mRNA editing elicited by early life stress occurs together with increased expression of Gαq mRNA and protein in vivo (Bhansali et al. 2007). Moreover, the downstream effector of Gαq, phospholipase C (PLC), is responsible for the formation of inositol 1,4,5-triphosphate (IP₃), which can be further phosphorylated to form inositol hexakisphosphate (IP₆). Interestingly, IP₆ is buried within the enzyme core of a prominent member of the editing enzyme family known as adenosine deaminases that act on RNA (ADARs), namely, ADAR2. Moreover, IP₆ has been

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shown to be required for ADAR2 editing activity and also for the activity of the related enzyme ADAT1 (which deaminates A37 of certain tRNAs) (Macbeth et al. 2005).

A second, yet unexplored, potential mechanism involves a change in enzymatic editing efficiency. Two ADARs (ADAR1 and ADAR2) edit the five exonic adenosines in 5-HT2C pre-mRNA, and they have both site-specific and overlapping roles: D-site editing is catalyzed by ADAR2 (Higuchi et al. 2000), A- and B-site editing requires ADAR1 (Wang et al. 2004), and C' and C-site editing requires both ADAR1 and ADAR2 (Hartner et al. 2004). Multiple alternatively spliced mRNA isoforms of ADAR1/2 have now been identified; from those, "a" and "b" mRNA isoforms of both enzymes are expressed in the brain. ADAR1b is a 5' splice variant of ADAR1a that has a 26 amino acid deletion within exon 7, which encodes the linker region between the third double-stranded RNA binding motif and the catalytic domain. In vitro, both ADAR1 splice variants differ in their efficiencies to edit 5-HT2C pre-mRNA, with ADAR1b having higher editing efficiency at the A- and C-sites (Liu et al. 1999). Conversely, "a" and "b" isoforms of ADAR2 have a less documented divergence in catalytic activity. Mouse ADAR2a lacks 10 amino acids that are found in the deaminase motif of ADAR2b (Melcher et al. 1996). In vitro, both isoforms of ADAR2 have indistinguishable editing activities on several sites of glutamate-receptor 2 pre-mRNA (Melcher et al. 1996), and it is presently not known whether their site-specific editing efficiencies of 5-HT2C pre-mRNA differ. Whether any of the known alternatively spliced isoforms of ADAR1/2 indeed play a significant role in inducing plastic changes in RNA editing in vivo remains to be elucidated.

Here, we show that sustained PLC activation increases mouse forebrain neocortical 5-HT2C pre-mRNA editing in vivo in an editing-site-specific manner, and that changes in alternative splicing of ADAR1 and ADAR2 pre-mRNA in vivo are also accompanied by changes in 5-HT2C-receptor editing. The site-specific changes detected in 5-HT2CR editing evoked by PLC activation only partially overlap with those exerted by a change in alternative ADAR pre-mRNA splicing.

RESULTS

The effect of PLC activation on 5-HT2C pre-mRNA editing

We have previously shown that a powerful mouse paradigm of early life stress, infant maternal separation (IMS) (Holmes et al. 2005; Priebe et al. 2005), elicits a persistent increase in 5-HT2CR editing as well as significantly increased Gαq mRNA and protein expression in the forebrain neocortex of the stress-susceptible mouse strain Balb/c (Bhansali et al. 2007). In contrast, as shown in Table 1, 5-HT2CR editing was unaltered in the stress-resilient strain C57Bl/6 when exposed to IMS.

TABLE 1. Forebrain neocortical 5-HT2C pre-mRNA editing in SFR, IMS, and cross-fostered (CF) IMS C57Bl/6 mice at P60

Editing	SFR	IMS	CF-IMS
Percent site-specific editing ^a			
A	90.8 ± 1.5	86.4 ± 3.5	85.6 ± 3.7
B	86.3 ± 0.5	78.5 ± 3.8	72.3 ± 7.1
C'	4.6 ± 1.1	3.2 ± 1.1	5.2 ± 1.1
C	24.8 ± 4.5	24.9 ± 1.4	41.6 ± 1.4 ^e
D	80.5 ± 2.6	75.0 ± 3.5	62.1 ± 2.9 ^e
Percent edited isoforms			
ABD ^b	51.6 ± 5.3	46.2 ± 2.7	24.2 ± 2.6 ^e
ABCD	19.7 ± 3.6	16.3 ± 1.4	23.1 ± 2.7
AB	9.1 ± 2.4	9.2 ± 1.9	13.2 ± 2.1
NE ^c	3.2 ± 0.5	3.7 ± 0.9	4.0 ± 1.5
AC/ABC ^d	2.3 ± 0.8	4.1 ± 1.2	11.5 ± 2.6 ^f

^aOne-way ANOVA revealed differences in C- ($F_{(2,11)} = 15.652$; $P = 0.0006$) and D-site editing ($F_{(2,11)} = 9.125$; $P = 0.005$) that were resolved post hoc using Tukey–Kramer Multiple Comparisons as indicated.

^bSignificant differences revealed by ANOVA ($F_{(2,11)} = 17.576$; $P = 0.0004$) and resolved post hoc are indicated.

^cNonedited.

^dSignificant differences revealed by ANOVA ($F_{(2,11)} = 6.738$; $P = 0.0123$) and resolved post hoc are indicated.

^e $P < 0.01$ compared with SFR and IMS mice.

^f $P < 0.05$ compared with SFR and IMS mice.

To further investigate the potential role of increased Gαq expression in modulating 5-HT2CR editing responses to IMS exposure, we first compared Gαq mRNA expression levels in the forebrain neocortex of nonstressed, standard facility reared (SFR), and IMS Balb/c and C57Bl/6 mice during postnatal development. Specifically, we examined Gαq mRNA expression levels at early postnatal ages (postnatal day 7 [P7], P15, and P21), during midadolescence and late adolescence (P35 and P45), and early adulthood (P60). Whereas IMS Balb/c mice expressed significantly higher Gαq mRNA expression than their SFR controls from P15 to adulthood (P60), IMS C57Bl/6 mice had increased Gαq mRNA expression only transiently from P15 to P21, having expression return to normal at P35. Moreover, a comparison between Gαq mRNA levels of IMS Balb/c and IMS C57Bl/6 mice, shown in Figure 1, revealed significantly higher Gαq mRNA expression in IMS Balb/c mice relative to IMS C57Bl/6 mice at P35, P45, and P60.

The sustained increase in Gαq expression in IMS Balb/c mice from mid-adolescence to adulthood prompted us to test whether sustained activation of the downstream effector, PLC, is responsible for the increased 5-HT2CR editing detected in these mice. Thus, we treated SFR Balb/c mice with the PLC activator *m*-3M3FBS for 3 d (see Materials and Methods). Results of real-time PCR experiments revealed that treatment with the PLC activator did not affect 5-HT2C mRNA expression levels (fold difference of *m*-3M3FBS-treated SFR Balb/c mice compared with controls: 1.29 ± 0.3) or expression of Gαq (fold difference of *m*-3M3FBS-treated SFR Balb/c mice compared with

FIGURE 1. Comparison of $G\alpha q$ mRNA expression in IMS C57Bl/6 and Balb/c mice during postnatal development. $G\alpha q$ mRNA levels (determined with real-time RT-PCR) are expressed as fold difference compared with SFR controls. Data are means $\pm$ sem ($n = 5$ –6 per group). For each developmental age, statistical differences between both groups of IMS mice were determined with two-tailed Student's *t*-tests.

controls: 1.30 ± 0.26). However, *m*-3M3FBS-treated mice exhibited altered 5-HT_{2C} editing. Specifically, *m*-3M3FBS treatment increased C- and D-site editing (Table 2). This led to increased expression of the ABD-edited mRNA isoform and decreased expression of AB-edited mRNA isoform (Table 2). Thus, while PLC activation did not alter editing at sites that are the sole targets for ADAR1 (A- and B-sites), sustained activation of PLC alone was sufficient to increase 5-HT_{2C} editing at sites that are either targeted solely by ADAR2 (D-site) or by both ADAR1 and ADAR2 (C-site).

Expression of alternatively spliced mRNA isoforms of ADAR1/2 during postnatal development of SFR and IMS Balb/c and C57Bl/6 mice

Since we have previously shown that IMS Balb/c mice exhibit increased 5-HT_{2C} editing not only at the C- and D-sites, but also A- and B-sites (Bhansali et al. 2007), increased PLC stimulation alone cannot account for the entire editing phenotype of IMS Balb/c mice. Hence, because the A and B editing sites are solely targeted by ADAR1, we further asked whether IMS Balb/c mice also differ in their expression in the alternatively spliced ADAR1 “a” and “b” isoforms.

We first measured the expression of mRNA encoding all known ADAR1 and ADAR2 mRNA isoforms in the forebrain neocortex. In real-time PCR experiments, “a” and “b” isoforms of ADAR1 and ADAR2 were reliably detected. Additional isoforms of ADAR2 described by Rueter et al. (1999), including autoedited ADAR2 transcripts containing a 47-nucleotide insertion at the 5′ end of the coding region, however, could not be detected in the same anatomic structure. We then measured the expression of “a” and “b” mRNA isoforms of ADAR1 and ADAR2 during postnatal development (P7–P60) of SFR and IMS Balb/c mice and compared the results with corresponding ones obtained from C57Bl/6 mice.

As shown in Figure 2, A and B, in P7 SFR Balb/c and C57Bl/6 mice, ADAR1a mRNA expression was about two-fold higher than the ADAR1b isoform, but, from P15 to

P60, both isoforms were expressed at equal levels. With the exception of a transiently higher expression of ADAR1a in P21 IMS Balb/c mice, the ratios of ADAR1a/1b expression were indistinguishable between SFR and IMS Balb/c mice (Fig. 2A). Surprisingly, however, in IMS C57Bl/6 mice, the P7 splicing pattern was maintained throughout postnatal development (P7–P60); i.e., mRNA encoding ADAR1a was twice as abundant as ADAR1b mRNA (Fig. 2B).

A similar result was obtained for ADAR2: In SFR Balb/c and C57Bl/6 mice, the relative expression of “a” and “b” ADAR2 isoforms follows the same postnatal developmental pattern detected for ADAR1, namely a ~ 2.5 -fold higher expression of ADAR2a relative to ADAR2b at P7 followed by equal expression of both isoforms between P15 and P60 (Fig. 2C,D). Moreover, both SFR and IMS Balb/c mice expressed roughly equal levels of ADAR2a/2b mRNA from P15 to P60 (Fig. 2C). However, just as the P7 alternative-splicing phenotype for ADAR1 was maintained throughout postnatal development in IMS C57Bl/6 mice, the same held true for ADAR2 (Fig. 2D).

In summary, while we did not detect a persistent change in the ratios of expression of alternatively spliced isoforms of ADAR1 and ADAR2 in IMS Balb/c mice, we found a change in these ratios in adolescent and adult IMS C57Bl/6 mice. At all ages examined in these mice, the expression of the “a” mRNA isoforms of ADAR1 and ADAR2 was significantly higher than their respective “b” isoforms. Since this change in alternative splicing of ADAR1 and ADAR2 pre-mRNA was not accompanied by altered 5-HT_{2C} editing (Table 1), the possibility that only a balanced (about equal) expression of “a” and “b” isoforms of ADAR1 and ADAR2 enables increased 5-HT_{2C}-editing responses to early life stress exposure was further tested. In these experiments, we took advantage of the fact that, when cross-fostered by Balb/c mothers at the time of birth, IMS

TABLE 2. Forebrain neocortical 5-HT_{2C} pre-mRNA editing in *m*-3M3FBS-treated SFR Balb/c mice

Editing	DMSO	<i>m</i> -3M3FBS
Percent site-specific editing		
A	66.0 $\pm$ 8.8	71.7 $\pm$ 5.7
B	59.5 $\pm$ 3.5	65.0 $\pm$ 4.2
C′	1.6 $\pm$ 1.6	1.1 $\pm$ 0.7
C	16.9 $\pm$ 4.8	44.8 $\pm$ 16.2*
D	46.2 $\pm$ 7.4	67.4 $\pm$ 8.3 *
Percent edited isoforms		
ABD	17.0 $\pm$ 3.1	31.7 $\pm$ 5.3***
ABCD	8.8 $\pm$ 1.8	11.5 $\pm$ 3.6
AB	29.2 $\pm$ 7.2	10.6 $\pm$ 5.2**
NE	15.9 $\pm$ 3.5	10.8 $\pm$ 2.9
AC/ABC	2.1 $\pm$ 1.4	6.3 $\pm$ 3.3
D	6.7 $\pm$ 2.1	10.4 $\pm$ 2.1

Significant differences revealed by two-tailed *t*-tests are indicated: (*) $P < 0.05$, (**) $P < 0.04$, (***) $P < 0.02$. (NE) Nonedited.

FIGURE 2. ADAR1a and ADAR2a mRNA expression levels relative to the expression of their b isoforms in SFR and IMS C57Bl/6 and Balb/c mice during postnatal development. (A) Ratios of ADAR1a/ADAR1b expression in SFR (white bars) and IMS (gray bars) Balb/c mice. (B) Ratios of ADAR1a/ADAR1b expression in SFR (black bars) and IMS (gray bars) C57Bl/6 mice. (C) Ratios of ADAR2a/ADAR2b expression in SFR (white bars) and IMS (gray bars) Balb/c mice. (D) Ratios of ADAR2a/ADAR2b expression in SFR (black bars) and IMS (gray bars) C57Bl/6 mice. mRNA expression levels were determined with real-time RT-PCR. (Dotted lines) Equal expression levels of “a” and “b” mRNA isoforms. Data are means $\pm$ sem ($n = 5$ –7 per group). Statistical differences between SFR and IMS mice of the same age group were determined with two-tailed Student’s *t*-tests.

C57Bl/6 mice will see their “a” and “b” ADAR1 and ADAR2 isoform expression revert back to normal (about equal) levels (Fig. 3A). Cross-fostered IMS C57Bl/6 mice, like *m*-3M3FBS-treated SFR Balb/c mice, did not exhibit increased expression of G α q mRNA (fold difference compared with controls: 1.23 ± 0.21), and Kruskal–Wallis nonparametric ANOVA also revealed no differences between cross-fostered SFR controls, IMS mice, and cross-fostered IMS mice (KW = 3.009; $P = 0.135$). However, cross-fostered IMS C57Bl/6 mice had altered 5-HT_{2C}R pre-mRNA editing: C-site editing was significantly increased, but D-site editing was significantly decreased. This led to decreased ABD-edited mRNA and increased AC/ABC-edited mRNA (Table 1). The decreased ABD-edited mRNA expression is substantially accounted for by increased expression of AC/ABC-edited mRNA, ABCD-edited mRNA, and mRNA edited only at the C-site (the latter was never found in SFR and non-cross-fostered IMS C57Bl/6 mice) (Table 1).

Altogether, the data summarized above illustrate two important differences between *m*-3M3FBS-treated Balb/c mice and cross-fostered C57Bl/6 mice. First, while increased C-site editing was found in *both* groups of mice, increased editing of the D-site (the exclusive target of ADAR2) was only found in Balb/c mice treated with the PLC activator. Second, in contrast to the effect of cross-fostering on ADAR1 and ADAR2 alternative pre-mRNA splicing in IMS C57Bl/6 mice, treatment with the PLC activator did not alter the normal alternative splicing pattern of ADAR1 and ADAR2 in Balb/c mice. In fact, neither the ratios of ADAR1a/1b

mRNA isoform expression (controls: 1.31 ± 0.38 ; *m*-3M3FBS-treated: 0.68 ± 0.24) nor the ratios of ADAR2a/2b isoform expression (controls: 0.72 ± 0.21 ; *m*-3M3FBS-treated: 0.90 ± 0.23) were affected by sustained PLC activation.

Expression of alternatively spliced mRNA isoforms of ADAR1 and ADAR2 in IMS Balb/c with diminished 5-HT_{2C}R editing responses to early life stress

The results obtained from studies on IMS C57Bl/6 mice suggest that changes in alternative ADAR1 and ADAR2 pre-mRNA splicing that favor “a” isoform expression of both enzymes are incompatible with increased 5-HT_{2C}R pre-mRNA editing responses to early life stress. To test this further, we examined ADAR1 and ADAR2 mRNA isoform expression in IMS Balb/c mice treated chronically with the antidepressant drug fluoxetine during adolescence (P32–P59). As reported earlier (Bhansali et al. 2007), adolescent fluoxetine treatment significantly diminished the 5-HT_{2C}R-editing responses to early life stress exposure in Balb/c mice. In contrast, adult fluoxetine treatment or raising mice in an enriched environment had no effect on 5-HT_{2C}R editing. Indeed, as shown in Figure 3, only adolescent fluoxetine treatment led to altered alternative splicing of ADAR1/2 pre-mRNA, and, just like IMS C57Bl/6 mice raised by their own mothers, IMS Balb/c mice treated with fluoxetine during adolescence expressed about twofold higher levels of ADAR1a and ADAR2a mRNA relative to their corresponding “b” isoforms (Fig. 3B). In

FIGURE 3. Effect of cross-fostering, fluoxetine, and environmental enrichment on ADAR1a and ADAR2a mRNA expression levels relative to the expression of their b isoforms. (A) Ratios of ADAR1 and ADAR2 a/b mRNA expression in SFR and IMS C57Bl/6 mice cross-fostered by Balb/c mothers compared with non-cross-fostered controls raised in parallel. These additional controls yielded results similar to those obtained for the other cohort shown in Figure 2. Note that cross-fostering of SFR mice did not alter the normal, about equal, ratios of ADAR1a/ADAR1b and ADAR2a/ADAR2b expression and that cross-fostering of IMS mice reverses the phenotype evoked by IMS alone. Data are means $\pm$ sem ($n = 7$ –9 per group). Kruskal–Wallis nonparametric ANOVA revealed significant differences between groups for the ratios of ADAR1a/ADAR1b ($P = 0.0256$) and ADAR2a/ADAR2b expression ($P = 0.0412$) that were resolved post hoc with Dunn’s Multiple Comparisons tests as indicated. (CF) Cross-fostered. (B) Ratios of ADAR1a/ADAR1b and ADAR2a/ADAR2b isoform expression in SFR and IMS Balb/c mice, and in IMS Balb/c mice treated with fluoxetine during adolescence (F32) or adulthood (F60), or exposed to an enriched environment (EE) during adolescence. Data are means $\pm$ sem ($n = 5$ per group). Kruskal–Wallis ANOVA revealed significant differences between groups for the ratios of ADAR1a/ADAR1b ($P = 0.038$) and ADAR2a/ADAR2b expression ($P = 0.039$) that were resolved post hoc with Dunn’s Multiple Comparisons tests as indicated.

contrast, adult fluoxetine treatment or adolescent environmental enrichment did not affect either ADAR1 or ADAR2 “a” over “b” mRNA isoform ratios (Fig. 3B). These findings support the conclusion that increased 5-HT_{2C}CR editing in response to early life stress is lost or diminished when “a” isoform expression dominates over “b” isoform expression for either enzyme.

DISCUSSION

In contrast to the persistent increase in 5-HT_{2C}CR editing in IMS Balb/c mice (Bhansali et al. 2007), we show here that the 5-HT_{2C}CR-editing phenotype of a different, stress-resilient

strain (C57Bl/6) is unaltered after the same IMS exposure. In addition, two other molecular phenotypes distinguish between the two strains of mice: Early life stress leads to increased G α q expression only in IMS Balb/c, and altered alternative splicing of ADAR1 and ADAR2 pre-mRNA occurs only in C57Bl/6 mice. Here, we began to investigate the functional relevance of altered G α q expression and/or altered alternative splicing of ADAR pre-mRNA in determining the 5-HT_{2C}CR-editing phenotypes that result from early life stress exposure.

The persistently increased expression of G α q that accompanies the increased editing of 5-HT_{2C}CRs in IMS Balb/c mice prompted us to test whether sustained activation of the downstream effector PLC alone would be sufficient to increase 5-HT_{2C}CR editing. Indeed, in SFR Balb/c mice, sustained activation of PLC led to increased editing at the C and D-sites. Since only D-site editing is solely mediated by ADAR2 (Higuchi et al. 2000) and C-site editing is catalyzed by either ADAR1 or ADAR2 (Hartner et al. 2004), it is not immediately apparent whether increased editing after PLC activation is due to increased editing efficiencies of both ADAR1 and ADAR2. However, a role of increased ADAR1-mediated editing at the C-site is unlikely since editing of the other, exclusive targets of ADAR1, namely the A- and B-sites, is unaltered after sustained PLC activation. Therefore, the data suggest that sustained PLC activation predominantly increases the catalytic activity of ADAR2. It is of interest to note that PLC activation leads to the formation of inositol phosphates whose successive phosphorylations ultimately lead to IP₆, an essential constituent of catalytically active ADAR2 protein and also required for the activity of the related enzyme ADAT1 (Macbeth et al. 2005). However, whether PLC activation can be directly linked to the catalytic activity of ADAR2, and whether or not IP₆ is also required for optimal enzymatic activity of ADAR1, requires further investigation. Since it is unlikely that IP₆ (which is deeply buried within the active site of ADAR2) can diffuse in and out of the protein in response to changing cellular environments, a sustained increase in IP₆ may be of relevance only for newly synthesized ADAR protein. This, however, would imply that cellular IP₆ levels are rate limiting for the expression of properly folded, catalytically active ADAR proteins. Hence, since IP₆ is present in the enzyme core of ADAR2, future studies can measure ADAR2 protein expression in cellular environments with different IP₆ levels and determine the amount of IP₆-containing ADAR2 relative to the inactive enzyme.

Although PLC stimulation increased 5-HT_{2C}CR editing at the C- and D-sites, Balb/c mice exposed to early life stress also have increased editing at the A- and B-sites (Bhansali et al. 2007), and these sites are solely targeted by ADAR1. Since “a” and “b” isoforms of ADAR1 have been shown to differ in their 5-HT_{2C}CR-editing efficiency *in vitro* (Liu et al. 1999), we further tested whether IMS Balb/c mice express different ratios of “a” over “b” isoforms compared with SFR

controls. However, we found that in the adolescent and adult forebrain neocortex, these ratios were indistinguishable between SFR and IMS Balb/c mice. In contrast, IMS C57Bl/6 mice exhibited changes in ADAR1 and ADAR2 alternative pre-mRNA splicing. In these mice, the normally occurring and developmentally programmed shift in alternative splicing of ADAR1 and ADAR2 pre-mRNA toward an equal expression of their “a” and “b” isoforms did not occur. In fact, the early postnatal alternative splicing pattern persisted throughout development; i.e., ADAR1a and ADAR2a mRNA isoforms remained expressed at significantly higher levels than their respective “b” isoforms during adolescence and adulthood. Since C57Bl/6 mice did not exhibit increased 5-HT2CR editing after IMS exposure, we reasoned that increased 5-HT2CR-editing responses to early life stress are lost when “a” isoform expression towers over “b” isoform expression. Two findings suggest that this is indeed the case: First, in IMS C57Bl/6 mice that were cross-fostered by Balb/c mothers, the normal expression of ADAR1 and ADAR2 “a” and “b” mRNA isoforms was restored, and these cross-fostered mice exhibited increased 5-HT2C receptor editing (Table 1). Specifically, C-site editing was significantly increased in these mice. In contrast to the stimulatory effect of PLC activation on D-site editing, however, D-site editing was decreased in cross-fostered IMS C57Bl/6 mice, indicating that the change in editing is not due to increased enzymatic activity of ADAR2. Rather, the data suggest that the altered ratio of “a” and “b” isoforms of ADAR1 (with the “b” isoform rising to the levels of the “a” isoform in cross-fostered mice) plays a significant role in mediating increased C-site editing. In vitro, ADAR1b has been shown to have higher catalytic activity at the A- and C-sites of 5-HT2CR pre-mRNA than ADAR1a (Liu et al. 1999). Since SFR C57Bl/6 mice already exhibited ~90% A-site editing at baseline, it was perhaps impossible to detect a further increase in cross-fostered IMS mice. Yet, increased C-site editing was indeed detected in these mice.

Of note, our findings also indicate that alternative splicing of ADAR pre-mRNA is not strictly determined by the genetic background. Rather, just like 5-HT2CR editing, alternative ADAR pre-mRNA splicing can also change in response to environmental stimuli. Not only can it be altered by fluoxetine treatment during adolescence, our cross-fostering study revealed that maternal care also has a persistent effect on this alternative splice-site selection. This is not surprising because, in rodent paradigms of early life stress, maternal behavior is a critical modulator of the offspring's behavioral and molecular responses to environmental stressors, and the maternal care of C57Bl/6 and Balb/c mothers differs substantially (that is, Balb/c mothers spend significantly less time tending to their pups) (Priebe et al. 2005).

In conclusion, alternative splicing of ADAR1 and ADAR2 pre-mRNA and activation of PLC have both overlapping and nonoverlapping roles in modulating the 5-HT2CR editing phenotypes in vivo. Moreover, while the present data

suggest that the role of ADAR pre-mRNA splicing may extend only to ADAR1, the effects of PLC activation clearly involve editing mediated by ADAR2. Nevertheless, the changes in 5-HT2CR editing following either sustained PLC activation or cross-fostering of IMS C57Bl/6 mice led to increased expression of 5-HT2CR mRNA encoding receptor proteins with a fourfold-lower ability to activate G protein (Werry et al. 2008) and, together, these two mechanisms can explain the altered 5-HT2CR-editing phenotype described for IMS Balb/c mice (Bhansali et al. 2007).

MATERIALS AND METHODS

Animals

All experiments involving animals were approved by the Institutional Animal Care and Use Committee at Columbia University. C57Bl/6J and Balb/cJ mice were housed in a facility with a 12-h light/dark cycle and ad libitum food and water.

Infant maternal separation (IMS)

Only offspring of first-time mothers were used in this study. Pups were separated from their mothers daily for 3 h from postnatal age P2–P15 as described previously (Bhansali et al. 2007). Standard facility reared offspring served as controls. Housing conditions, including cage-cleaning schedules, were identical for both groups, and males and females were equally represented in each group. SFR and IMS pups were killed by rapid decapitation at P7, P15, or P21, their brains were removed, and forebrain neocortical tissues were dissected. The remaining animals were weaned at P28 and group housed by sex until they were killed at P35, P45, or P60; their brains were processed as described above.

Cross-fostering

At the time of birth, C57Bl/6 pups were immediately transferred to pariturient Balb/c mothers and assigned to either IMS or SFR groups. They were also weaned at P28 and group-housed until P60. There was no difference in the body weights between male and female cross-fostered SFR and IMS mice at P60, and three developmental milestones (disappearance of milk spots, ear detachment, and eye opening) were reached at the same time.

Drug treatments

The compound 2,4,6-trimethyl-*N*-(*meta*-3-trifluoromethylphenyl)-benzenesulfonamide (*m*-3M3FBS) was dissolved in DMSO and subsequently diluted in drinking water (containing 2% sucrose), and mice consumed ~10 mg/kg/day of *m*-3M3FBS. Control animals received drinking water containing 2% sucrose and the same amount of DMSO that *m*-3M3FBS-treated animals received. *m*-3M3FBS directly activates PLC (with no isoform specificity) and has been shown to increase the formation of inositol phosphates (Bae et al. 2003). Moreover, systemic administration of the compound to mice (5 mg/kg) has been shown to produce significant effects on PLC activation (Fioravanti et al. 2008).

The procedure for chronic treatment with fluoxetine hydrochloride (16 mg/kg/day; Sigma Aldrich) and housing mice in enriched environment was previously described (Bhansali et al. 2007).

RNA extraction, real-time reverse transcription PCR, and nucleotide sequencing

RNA was extracted using guanidine/cesium chloride ultracentrifugation. First-strand cDNA was synthesized using murine moloney leukemia virus reverse transcriptase (USB). Real-time PCR was performed using the iQ Real Time PCR detection System (Bio-Rad) and SYBR Green (Bio-Rad). For amplification of ADAR1 a/b, isoform-specific 5' primers (1a: 5'-CCAAAGACACTTCCTCTC-3'; 1b: 5'-GCAGAGCTTCCTCTCTCT-3') were used in conjunction with a common 3' primer (5'-CAGTGTGGTGGTTGTACT-3'). Similarly, ADAR2 "a" and "b" isoforms were amplified with isoform-specific 5' primers (2a: 5'-CTAGAGGAACCAGCAGAT-3'; 2b: 5'-CTGACAGAACCAGCAGAT-3') and a common 3' primer (5'-GGTCTCTATGTTGGAGA-3'). Cycle thresholds (Ct) of amplification (normalized to β -actin whose Ct values did not differ between groups) were expressed as $2^{\Delta C_t}$ values. The ratios of "a" over "b" isoforms were calculated ($2^{\Delta\Delta C_t}$) and expressed as fold difference. G α_q cDNA was amplified using the primer pair 5'-ACTCTGGAGTCCATCATG-3'/5'-TGTATGGGATCTTGAGCG-3'. Ct values (also normalized using Ct values obtained for β -actin) were calculated using the $2^{\Delta C_t}$ method and expressed as fold difference (SFR versus IMS mice).

5-HT_{2C} cDNA was amplified by PCR, and gel-purified PCR products were cloned into the pCRII cloning vector (Invitrogen) as previously described (Bhansali et al. 2007). For each animal, the nucleotide sequence of 5-HT_{2C} cDNA inserts was obtained from plasmid DNA extracted from 36–40 transformed bacterial colonies using Sanger's dideoxy chain-termination method.

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REFERENCES

- Bae Y-S, Lee TG, Park JC, Hur JH, Kim Y, Heo K, Kwak J-Y, Suh P-G, Ryu SH. 2003. Identification of a compound that directly stimulates phospholipase C. *Mol Pharmacol* **63**: 1043–1050.
- Bass BL. 2002. RNA editing by adenosine deaminases that act on RNA. *Annu Rev Biochem* **71**: 817–846.
- Bhansali P, Dunning J, Singer SE, David L, Schmauss C. 2007. Early life stress alters adult serotonin 2C receptor pre-mRNA editing and expression of the α subunit of the heterotrimeric G-protein G $_q$. *J Neurosci* **27**: 1467–1473.
- Englander MT, Dulawa S, Bhansali P, Schmauss C. 2005. How stress and fluoxetine modulate serotonin 2C receptor pre-mRNA editing. *J Neurosci* **25**: 648–651.
- Fioravanti B, DeFelice M, Stucky CL, Medler KA, Luo M-C, Gardell LR, Ibrahim M, Malan TP Jr, Yamamura MH, King T, et al. 2008. Constitutive activity at the cannabinoid CB1 receptor is required for behavioral response to noxious chemical stimulation of TRPV1: Antinociceptive actions of CB1 inverse agonists. *J Neurosci* **28**: 11593–11602.
- Gurevich I, Englander MT, Adlersberg M, Siegal N, Schmauss C. 2002. Modulation of serotonin 2C receptor editing by sustained changes in serotonergic neurotransmission. *J Neurosci* **22**: 10529–10532.
- Hartner JC, Schmittwolf C, Kispert A, Mueller AM, Higuchi M, Seeburg PH. 2004. Liver disintegration in the mouse embryo by deficiency in RNA editing enzyme ADAR1. *J Biol Chem* **279**: 4894–4902.
- Higuchi M, Maas S, Single FN, Hartner J, Rozov A, Burnashev N, Feldmeyer D, Sprengel R, Seeburg PH. 2000. Point mutation in an AMPA receptor gene rescues lethality in mice deficient in the RNA-editing enzyme ADAR2. *Nature* **406**: 78–81.
- Holmes A, le Guisquet AM, Vogel E, Millstein RA, Leman S, Belzung C. 2005. Early life genetic, epigenetic, and environmental factors shaping emotionality in rodents. *Neurosci Biobehav Rev* **29**: 1335–1346.
- Levanon EY, Eisenberg E, Yelin R, Nemzer S, Hallegger M, Shemesh R, Fligelman ZY, Shoshan A, Pollock SR, Szybel D, et al. 2004. Systematic identification of abundant A-to-I editing sites in the human transcriptome. *Nat Biotechnol* **22**: 1001–1005.
- Li JB, Levanon EY, Yoon J-K, Aach J, Xie B, LeProust E, Zhang K, Gao Y, Church GM. 2009. Genome-wide identification of human RNA editing sites by parallel DNA capturing and sequencing. *Science* **324**: 1210–1213.
- Liu Y, Emeson RB, Samuel CE. 1999. Serotonin 2C receptor pre-mRNA editing in rat brain and in vitro by splice-site variants of the interferon-inducible double-stranded RNA-specific adenosine deaminase ADAR1. *J Biol Chem* **274**: 18351–18358.
- Macbeth MR, Schubert HL, VanDemark AP, Lingam AT, Hill CP, Bass BL. 2005. Inositol hexakisphosphate is bound in the ADAR2 core and required for RNA editing. *Science* **309**: 1534–1539.
- Melcher T, Maas S, Herb A, Sprengel R, Higuchi M, Seeburg PE. 1996. RED2, a brain-specific member of the RNA-specific adenosine deaminase family. *J Biol Chem* **271**: 31795–31798.
- Priebe K, Brake WG, Romeo RD, Sisti HM, Mueller A, McEwen BS, Francis DD. 2005. Maternal influence on adult stress and anxiety-like behavior in C57Bl/6J and BALB/CJ Mice: A cross fostering study. *Dev Psychobiol* **47**: 398–407.
- Rueter SM, Dawson TR, Emeson RB. 1999. Regulation of alternative splicing by RNA editing. *Nature* **399**: 75–80.
- Wang Q, Miyakoda M, Yang W, Khillan J, Stachura DL, Weiss MJ, Nishikura K. 2004. Stress-induced apoptosis associated with null mutation of ADAR1 RNA editing deaminase gene. *J Biol Chem* **279**: 4952–4961.
- Werry TD, Loiacono R, Sexton PM, Christopoulos A. 2008. RNA editing of the serotonin 5HT_{2C} receptor and its effects on cell signaling, pharmacology and brain function. *Pharmacol Ther* **119**: 7–23.

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