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Altered ADAR2 equilibrium and $5HT_{2C}R$ editing in the prefrontal cortex of ADAR2 transgenic mice

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Abstract

Modulation of serotonin signaling by RNA editing of the serotonin 2C receptor ($5HT_{2C}R$) may be relevant to affective disorder as serotonin functions regulate mood and behavior. Previously we observed enhanced endogenous behavioral despair in ADAR2 transgenic mice. Since the transcript of the $5HT_{2C}R$ is a substrate of ADAR2, we hypothesized that perturbed ADAR2 equilibrium in the prefrontal cortex of ADAR2 transgenic mice alters the normal distribution of edited amino acid isoforms of the $5HT_{2C}R$ and modifies the receptor function in downstream basal ERK signaling. We examined groups of naïve control and ADAR2 transgenic mice and found significantly increased ADAR2 expression, increased RNA editing at A, C, D and E sites and significantly altered normal distribution of edited amino acid isoforms of the $5HT_{2C}R$ with increased proportions of VNV, VSV, VNI, INV and decreased INI amino acid isoforms of the $5HT_{2C}R$ in ADAR2 transgenic mice. Localized serotonin levels (5-HT) were unchanged and perturbed ADAR2 equilibrium coincides with dysregulated edited amino acid isoforms of the $5HT_{2C}R$ and reduced basal ERK signaling. These results altogether suggest that altered $5HT_{2C}R$ function could be contributing to enhanced depression-like behavior of ADAR2 transgenic mice and further implicate ADAR2 as a contributing factor in cases of affective disorder.

Keywords

RNA editing; behavioral despair; ADAR2; ERK signal and $5HT_{2C}R$

INTRODUCTION

ADAR2 (*ADARB1*) is an RNA editing enzyme, which belongs to ADAR family of enzymes that catalyzes the conversion of adenosine to inosine (A-to-I) in pre-mRNA. ADAR2 auto edits its own transcript and regulates the ADAR2 protein expression (Feng *et al.*, 2006, Rueter *et al.*, 1999). A-to-I RNA editing occurs in several receptors and ion channels such as $5HT_{2C}R$, GluRB subunit of AMPA receptor, α3 subunit of the $GABA_A$ receptor and the

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KV1.1 potassium channel (Burnashev *et al.*, 1992, Burns *et al.*, 1997, Ohlson *et al.*, 2007, Schmauss & Howe, 2002, Sommer *et al.*, 1991). Amongst all these substrates the $5HT_{2C}R$ is implicated in the pathophysiology of schizophrenia, bipolar disorder, anxiety and major depression (Frey *et al.*, 2007, Gurevich *et al.*, 2002b, Iwamoto & Kato, 2003, Lan *et al.*, 2009, Niswender *et al.*, 2001, Pilc *et al.*, 2008, Sodhi *et al.*, 2001).

The potential for dysregulated ADAR2 expression in aberrant RNA editing of the $5HT_{2C}R$ in ADAR2 transgenic mice was examined as they display several endocrine and behavioral changes that has been associated with the $5HT_{2C}R$ and affective disorder, such as elevated endogenous behavioral despair, anxiety-like behavior and a hyperactive hypothalamic pituitary-adrenal-axis (HPA) (Singh *et al.*, 2009).

Reports from both humans and rodent models of depression suggest that abnormal RNA editing of the $5HT_{2C}R$ is associated with depression (Dracheva *et al.*, 2003, Fitzgerald *et al.*, 1999, Gardiner & Du, 2006, Gurevich *et al.*, 2002b, Iwamoto *et al.*, 2005a, Schmauss, 2003, Yang *et al.*, 2004), anxiety (Hackler *et al.*, 2006) and Prader Willi-like syndrome (Morabito *et al.*, 2010a). Both serotonin and antidepressants also alter RNA editing of the $5HT_{2C}R$ (Fitzgerald *et al.*, 1999, Gardiner & Du, 2006, Gurevich *et al.*, 2002a, Schmauss & Howe, 2002, Tohda *et al.*, 2006).

Central serotonergic neurotransmission is mediated by at least fourteen receptor subtypes, out of which, the $5HT_{2C}R$ is the only one presently known to undergo A-to-I RNA editing, catalyzed by both ADAR1 and ADAR2 that generates 24 amino acid isoforms of the $5HT_{2C}R$, each with their distinct signaling properties (Burns *et al.*, 1997, Wang *et al.*, 2000, Werry *et al.*, 2008a, Werry *et al.*, 2008b). The altered amino acid sequence of $5HT_{2C}R$ by RNA editing leads to reduced G protein coupling, reduced phospholipase C signaling (Burns *et al.*, 1997) and altered phosphorylation of ERK signal (Labasque *et al.*, 2010, Werry *et al.*, 2008a, Werry *et al.*, 2008b)

Altered 5-HT signaling and altered RNA editing of the $5HT_{2C}R$ in the prefrontal cortex region is associated with depression (Englander *et al.*, 2005, Iwamoto & Kato, 2003, Iwamoto *et al.*, 2005b, Meltzer, 1989, Schmauss, 2003) and therefore RNA editing changes of the $5HT_{2C}R$ may be relevant to depression-like behavior of ADAR2 transgenic mice. ERK signal participates in stress response and stress induces depressive-like behavior (Qi *et al.*, 2008). Depression is ameliorated by brain derived neurotropic factor (BDNF) *via* activating the ERK pathway (Shirayama *et al.*, 2002). Serotonin treatments induce extracellular signal-regulated kinase (ERK) phsophorylation (Lee *et al.*, 2001) and cyclic AMP-responsive-element-binding protein (CREB) is a downstream target of ERK (Hardingham *et al.*, 2001, Martinowich & Lu, 2008, Qi *et al.*, 2008, Ying *et al.*, 2002). RNA editing of the $5HT_{2C}R$ is linked to ERK signaling and mood disorder (Dracheva *et al.*, 2008a, Gurevich *et al.*, 2002b, Iwamoto & Kato, 2003, Niswender *et al.*, 2001, Werry *et al.*, 2005, Werry *et al.*, 2008a, Werry *et al.*, 2008b). These results altogether suggest that ERK pathway is the major convergence point in all signal pathways of mood disorder and RNA editing could play a critical role in behaviors of ADAR2 transgenic mice.

In this study, the prefrontal cortex region from naïve control and ADAR2 transgenic mice was used to examine RNA editing changes of the $5HT_{2C}R$, compensatory changes in gene expression of ADAR family, expression changes in genes $5HT_{2C}R$, BDNF and CREB. We also examined the steady state protein levels of ADAR2, $5HT_{2C}R$ and reduced function of the $5HT_{2C}R$ on the downstream ERK signaling in control and ADAR2 transgenic mice.

MATERIALS AND METHODS

Animal husbandry

Animals were housed in individual plastic cages with cellular bedding. Rodent chow (7013 NIH-31 Modified diet), and tap water were available *ad libitum* for the duration of the experiments. All studies were conducted on ADAR2 transgenic or wild-type mice (e.g., control littermates) that were maintained on a B6D2F1 hybrid background by back-crossing to either a male or a female B6D2F1 mouse (Singh *et al.*, 2007). The temperature in the mice colony room was maintained at 22°C. The light cycle was held at 12:12 h, with lights on at 0600 h. All procedures were approved and conducted in accordance with the University of Iowa Institutional Animal Care and Use Committee.

Genotyping

For genotype screening, sense and antisense primers corresponding to transgene were used to PCR amplify the transgene from the genomic DNA isolated from 10 μl of whole blood (obtained from tail snips) of control and transgenic mice using the RED Extract-N-Amp Blood PCR Kit (Sigma-Aldrich, St. Louis, MO) (Singh *et al.*, 2007).

Animals used in this study

ADAR2 transgenic mouse misexpresses the active rat ADAR2b cDNA under the control of cytomegalovirus (CMV) promoter (Singh *et al.*, 2007). Previously we observed that ADAR2 transgenic mice misexpressing the active ADAR2b enzyme displayed enhanced behavioral despair in both sexes without the use of chronic stress method that is typically used to induce depression-like behavior in rodent model of depression (Singh *et al.*, 2009) and therefore ADAR2 transgenic mouse has been referred to as a rodent model of enhanced endogenous behavioral despair (Singh *et al.*, 2009). Complex RNA editing changes from rodent models of depression have been previously reported using groups of 4 or more mice indicating that RNA editing changes in the $5HT_{2C}R$ are homogenous in nature amongst animals to detect significant differences in a small group of animals (Englander *et al.*, 2005, Iwamoto *et al.*, 2005b). In this study we examined molecular changes in the prefrontal cortex region of two groups of naïve control and transgenic mice (n=4) and all our results of ADAR2 transgenic mice have been compared with their control littermates.

Quantitative real time PCR analysis of gene expression in the prefrontal cortex of control and transgenic mice

The prefrontal cortex region was isolated on a cryostat and total RNA was isolated from the prefrontal cortex by using TRIzol reagent (Invitrogen, Carlsbad, CA). One μg of total RNA from each animal was reverse transcribed with random hexamers (Roche Diagnostic Corp., Chicago, IL) into cDNA using SuperScript II Reverse Transcriptase (Invitrogen). The cDNA was subsequently used for quantitative PCR. Quantitative real time PCR experiments were carried out in a Bio-Rad iCycler (Bio-Rad Laboratories, Los Angeles, CA) using the fluorogenic intercalating dye SYBR green and gene specific primers of mouse or rat. For each gene of interest; CREB, BDNF, ADAR2, ADAR1 and ADAR3, the forward and reverse mouse specific primers were used (Table 1). Each 25 μl reaction contained 2 mM $MgCl_2$, 1X PCR buffer, 0.65 μM each primer, 1.25 units Taq Polymerase, 5 μl cDNA and 2.5 μl SYBR green PCR master mix. PCR parameters were at an initial penetration at 95°C for 60s, followed by 35 cycles of 95°C for 20s, 60°C for 20s and 72°C for 20s. Relative gene expression was normalized with GAPDH expression. The cycle of amplification threshold (CT) was assessed, and the melting curve was used to verify the identity of the amplification products. The CT difference between the housekeeping gene GAPDH and the gene of interest for every sample (ΔCT) was determined.

Western blot analysis

Total cellular lysate from the prefrontal cortex was prepared using modified RIPA buffer [100 mM NaCl, 1% IGPAL CA 630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 7.6 with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 0.1% aprotinin; Sigma-Aldrich). Protein concentration was determined by BCA reagents (Thermo Scientific, Rockford, IL) and 20 μg of total protein from each mouse sample was resolved by denaturing polyacrylamide gel electrophoresis (10% SDS-PAGE) and transferred to a nitrocellulose membrane and rabbit anti-ADAR2-specific antiserum (1:1000 Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-5-HT_{2C}R (1:500, Santa Cruz Biotechnology), rabbit anti-ERK (1:2000, cell signaling Technology Inc., Danvers, MA), rabbit anti-pERK (1:1000, cell signaling Technology Inc.), and rabbit-anti-β actin antiserum (1:2500 Sigma-Aldrich, St Luis, MO), was used as a control for normalizing loading for each sample (Dwivedi *et al.*, 2001, Pandey *et al.*, 2006, Singh *et al.*, 2007, Werry *et al.*, 2005). Anti-rabbit Alexa Fluor 680 (1:10,000 [vol/vol], Molecular Probes, Eugene, OR) was used as the secondary antibody. The secondary antibody was detected directly using an Odyssey infrared imaging system in accordance with the manufacturer's instructions (LI-COR Biosciences, Lincoln, NE) (Feng *et al.*, 2006, Singh *et al.*, 2007)

Immunohistochemistry

Control and ADAR2 transgenic mice were perfused intracardially first with 0.1 M phosphate buffer and then with 4% paraformaldehyde. Brains were extracted and post-fixed with 4% paraformaldehyde and cryoprotected in 30% sucrose. They were then cut on a freezing microtome at 40 μm and processed for ADAR2-immunoreactivity. Briefly, brain sections were soaked in 3% hydrogen peroxide and 10% methanol solution. The sections were rinsed in PBS, and then placed in normal goat serum solution. Sections were rinsed again with PBS before being placed in the primary antibody solution for 1 hr (rabbit anti-ADAR2 polyclonal IgG 1:5000, Santa Cruz Biotechnology). The brain sections were rinsed thoroughly with PBS, incubated in a secondary antibody overnight (anti-rabbit biotinylated IgG 1:200, Vector Laboratories, Burlingame, CA), and then rinsed again and soaked in PBS. The sections were reacted with Avidin–Biotin complex (Vector Laboratories), and then rinsed with PBS before a reaction with 0.02% diaminobenzidine solution. Hydrogen peroxide (0.01%) was used to initiate the reaction. Immediately following staining, the reaction was stopped by rinsing with PBS. Sections were mounted on slides and cover slipped with Permount (Fisher Scientific, Fairlawn, NJ) (Grippo *et al.*, 2004).

RNA editing analysis of the prefrontal cortex of control and transgenic mice

PCR amplification of 5HT_{2C}R RNA edited region—RNA isolated using the previous protocol was used to synthesize cDNAs. The cDNAs were subsequently used for further characterization of edited transcripts. PCR amplification of the 5HT_{2C}R RNA edited region was performed using 1 mM concentration of each forward 5’TTTCAACTGCGTCCATCATGCACCT3’ and reverse primer 5’AACGAAGTTGGGGTCATTGAGCAC3’, 250 mM of dATP, dCTP, dGTP, and dTTP (dNTPs), 2 units of DNA polymerase and recommended buffer (Qiagen, Valencia, CA) in a total volume of 20 μl. Samples were amplified for 27 cycles; cycling conditions comprised 1 cycle at 96°C for 5 minutes followed by 27 cycles at 96°C for 15s, 50°C for 30s and 72°C for 30s, and finally 1 cycle of 72°C for 5 minutes in a thermal cycler (Bio-Rad Laboratories) resulting in 236bp amplicon. The 236bp PCR amplicon was gel purified using Qiaex II (Qiagen).

Characterization of RNA edited transcripts—The gel purified 236bp amplicon was subcloned in pGEM vector (Promega, Madison, WI). At least 45 individual colonies from

each animal was picked and directly inoculated into the 30 μl PCR mixture containing; 0.2 μM sense oligonucleotide primer 5'ATATCGCTGGATCGGTATGTAG3' and 0.2 μM antisense biotinylated primer 5'BIOTIN CGAATTGAAACGGCTATGCT3', 1X Taq Gold buffer with 1.5 mM $MgCl_2$ (PerkinElmer Life and Analytical Sciences, Shelton, CT), 0.75 units of Taq Gold (PerkinElmer) and 0.24 mM dNTPs and subjected to colony PCR. PCR was performed in a thermal cycler using the following conditions: 96°C for 5 minutes, then amplified for 30 cycles each consisting of 20s at 96°C, 30s at 58°C, and 20s at 72°C in a thermal cycler (Bio-Rad Laboratories). Five μl of the amplified single product (67bp) were electrophoresed on a 3% agarose gel to verify a single 67bp amplicon. The remaining 25 μl PCR product was subjected to high throughput pyrosequencing following the Biotage ABI based paradigm (Qiagen, Valencia, CA) (Hackler *et al.*, 2006, Singh *et al.*, 2007, Sodhi *et al.*, 2005).

HPLC Methods for Biogenic amines

Tissue Extraction—The prefrontal cortex region was homogenized, using a tissue dismembrator, in 100-750 ul of 0.1M TCA, which contains 10^{-2} M sodium acetate, 10^{-4} M EDTA, 5ng/ml isoproterenol (as internal standard) and 10.5 % methanol (pH 3.8). Samples were spun in a micro centrifuge at 10000 g for 20 minutes. The supernatant was removed and stored at –80°C. The pellet was saved for protein analysis. Supernatant was then thawed and spun for 20 minutes. Samples of the supernatant were then analyzed for biogenic monoamines by HPLC method.

Biogenic amines—Biogenic amines were determined by a specific HPLC assay utilizing an Antec Decade II (oxidation: 0.5) electrochemical detector operated at 33° C. Twenty μl samples of the supernatant were injected using a Water 717+ auto sampler onto a Phenomenex Nucleosil (5u, 100A) C18 HPLC column (150 × 4.60 mm). Biogenic amines were eluted with a mobile phase consisting of 89.5% 0.1M TCA, 10^{-2} M sodium acetate, 10^{-4} M EDTA and 10.5 % methanol (pH 3.8). Solvent was delivered at 0.6 ml/min using a Waters 515 HPLC pump. Using this HPLC solvent the following biogenic amines elute in the following order: Noradrenaline, Adrenaline, DOPAC, Dopamine, 5-HIAA, HVA, 5-HT, and 3-MT. HPLC control and data acquisition were managed by Enpower 2 software (Vanderbilt Neurogenomics core lab, Vanderbilt University).

Protein—Total protein concentrations of prefrontal cortex extracts were determined using BCA Protein Assay Kit purchase from Pierce Chemical Company (Rockford, IL). The frozen pellets were allowed to thaw and reconstituted in a volume of 0.5 N HCl that equals that previously used for tissue homogenization. On hundred μl of this solution was combined with 2 ml of color reagent and allowed to develop for 2 hours. A BSA standard curve was run at the same time spanning the concentration range of 20-2000 μg/ml. Absorbance's of standard and samples were measured at 562 nm.

Statistical analyses—For quantitative real time PCR analysis, statistical analyses were performed on relative fold-changes determined by the ΔΔCT method and student *t*-tests were used to evaluate the significant difference of ΔΔCT values between transgenic (n=4) and control mice (n=4).

To test the effect of ADAR2 on the editing frequency of $5HT_{2C}R$ in each of the 5 sites within the prefrontal cortex, logistic regression fitted by the method of generalized estimating equations (GEE) was used. The logistic regression model included as independent variables genotype (control mice vs. ADAR2 transgenic mice). The GEE method was used in fitting the logistic regression model to account for the correlation of site editing between clones from the same animal. The same analysis was performed to test for

differences in amino acid isoform expression between ADAR2 transgenic and control mice. Independence of neighboring sites in transgenic and control mice was tested using the Pearson chi-square test. Independence of neighboring sites in transgenic and control mice was also tested using logistic regression fitted by the GEE method. This analysis was used for all neighboring site pairs except for A and B sites where there were zero cell counts for which computations for fitting a logistic model cannot be performed. For the A-B site pair, Pearson's Chi-square and Cochran-Mantel-Haenszel Chi-square were used.

RESULTS

Altered behavioral despair without altered serotonin levels in ADAR2 transgenic mice

ADAR2 transgenic mice misexpress ADAR2 cDNA under the control CMV promoter and display enhanced behavioral despair without any experimental manipulation. Therefore termed as endogenous behavioral despair (Singh *et al.*, 2009). In this study we determined the transgene expression from the prefrontal cortex tissue of previous studied ADAR2-Tg mice with enhanced endogenous behavioral despair (Singh *et al.*, 2009). RT-PCR on RNA samples and gel electrophoresis of PCR products confirmed that the transgene was expressed in the prefrontal cortex of ADAR2 transgenic mice (Fig. 1a).

HPLC analysis of biogenic amines showed no changes in serotonin, dopamine and noradrenaline levels in the prefrontal cortex of ADAR2 transgenic mice when compared to their control littermates ($p>0.05$, Fig 1b). These results suggest that behavioral despair in ADAR2 transgenic mice is independent of serotonin levels.

Increased ADAR2 mRNA in the prefrontal cortex of ADAR2 transgenic mice

The total ADAR2 mRNA expression (mouse and rat) was significantly increased in the prefrontal cortex of ADAR2 transgenic mice when compared to control littermates ($P<0.001$, Fig. 2A).

Qualitative immunohistochemistry showed increased ADAR2 protein expression in the prefrontal cortex of ADAR2 transgenic mice when compared to control littermates (Fig. 3A).

Quantitative western analysis showed increased ADAR2 protein expression by 1.4±0.36 fold more in the prefrontal cortex of ADAR2 transgenic mice compared to control littermates (Fig. 3 B &C).

Gene expression analysis showed no changes in ADAR1 and ADAR3 mRNA expression as well as no changes in BDNF, CREB and $5HT_{2C}R$ mRNA expressions when compared to their control littermates (Fig. 2A and B).

$5HT_{2C}R$ protein expression is unchanged in the prefrontal cortex of ADAR2 transgenic mice

Western blot analysis showed no change in $5HT_{2C}R$ protein expression in the prefrontal cortex of ADAR2 transgenic mice when compared to their control littermates (Fig.4A and B).

Distinct RNA editing changes of $5HT_{2C}R$ pre-mRNA in the prefrontal cortex of ADAR2 transgenic mice

Pyrosequencing analysis of 67bp PCR amplicon showed a significant increased editing at A ($p<0.002$), C ($p<0.0009$), D ($p<0.0001$) and E ($p<0.042$) sites in ADAR2 transgenic mice when compared to their control littermates (Table 2). The clones from ADAR2 transgenic

mice were more likely to exhibit $5HT_{2C}R$ mRNA editing at these sites compared to control mice with odds ratio of 2.08 (95% CI: 1.32, 3.28) in edited site A, 1.60 (1.21, 2.11) in site C, 2.40 (2.20, 2.62) in site D and 7.85 (1.08, 57.09) in site E. The variation in the editing levels present in $5HT_{2C}R$ transcripts was addressed also by determining total number of inosine residues. ADAR2 transgenic mice showed a significantly higher proportion of $5HT_{2C}R$ transcripts with 3 or more inosine residues ($P<0.0001$; odds ratio: 1.97) and 4 or more inosine residues when compared to control mice ($p<0.0001$; odds ratio: 1.54; Table 3).

Altered RNA editing site (independence from neighboring sites) in ADAR2 transgenic mice

All 5 sites that are modifiable are closely spaced within the $5HT_{2C}R$ transcript. The possibility of one site editing and influencing the other or independent regardless of editing status was examined.

Linkage at A and B sites—Editing at A and B sites were not independent in both control and ADAR2 transgenic mice. The probability of A site editing was significantly higher when the B site was edited in both control and transgenic mice [Table 4. B (+) vs. (B-) Fisher's exact test $p<0.0001$] with a greater probability of A site editing seen in the transgenic group compared to control (Chi-square=11.47; $p<0.0007$). When the A site was not edited, the probability of editing at the B site was significantly lower in transgenic mice when compared to control mice (transgenic, 31.2% vs. control, 68.1%; Chi-square=6.68; $p<0.010$).

Linkage at E and B sites—No evidence of linkage was observed between the E and B sites in either transgenic or control mice. However, in ADAR2 transgenic mice, we did find that when the B site was edited, there was significantly greater E site editing compared to their control littermates (Fisher's exact test, $p<0.038$).

Linkage at E and C sites—A linkage was observed at E and C site editing in ADAR2 transgenic mice (Fisher's exact test $p<0.05$) with a significantly higher probability of E site editing when the C site was edited. However, similar linkage at E and C site editing was not observed in control mice.

Linkage at C and D sites—In control mice, the probability of D site editing was significantly greater in the adjacent C edited site versus the C non-edited site (Chi square =16.15, $p<0.0001$; 75.7% vs. 40.3%). These results indicate that there is a linkage at C and D sites observed in control mice. Similar pattern was observed in transgenic mice with smaller differences in the probability of D site editing (or C site editing) in the context of edited versus non-edited adjacent site. This showed a significant interaction effect between genotype and editing in the neighboring site (Wald Chi square=16.42, $p<0.0001$), indicating that the degree of linkage is significantly favorable in control mice compared to ADAR2 transgenic mice.

Altered amino acid isoforms of the $5HT_{2C}R$ in the prefrontal cortex of naïve transgenic mice

The clones from the ADAR2 transgenic mice had a significantly higher proportion of sequences coding for the $5HT_{2C}R$ isoforms with VNV, VSV, INV and other grouped minor amino acid isoform expression (VNV: transgenic = 45.3% vs. control = 34.5%; $P<0.0001$; VSV: transgenic=13.3% vs. control 10.2%, $p<0.0001$; INV: transgenic=5.0% vs. 0.4%, $p<0.003$; grouped minor amino acid isoforms: transgenic = 5.5% vs. 0.4%, $p<0.007$) when compared to control mice (Table 5). The odds ratio of VNV isoforms in transgenic mice relative to control mice was 1.57 (confidence interval = 95%; transgenic = 1.36, control = 1.82), and VSV isoform odds ratio 1.35 (confidence interval=95%; transgenic= 1.20, control

=1.51), INV isoform odds ratio 14.34 (confidence interval=95%; transgenic =2.53, control =81.2) and grouped minor isoform odds ratio 16.02 (confidence interval =95%; transgenic = 2.16, control = 118.6). The ADAR2 transgenic mice expressed a lower proportion of the VNI and INI isoforms compared to control mice (VNI: transgenic = 26% vs. control = 34.5%, $p<0.0001$; INI: transgenic = 0.6% vs. control =5.1%, $p<0.012$). The odds ratio of VNI and INI isoforms in transgenic mice relative to control were (VNI: 0.66, tr= 0.58, co = 0.76; INI: 0.10. tr =0.02, co = 0.61).

Altered ERK signal in the prefrontal cortex of naïve ADAR2 transgenic mice

Western analysis showed significant reduction of basal ERK phosphorylation in the prefrontal cortex of ADAR2 transgenic mice when compared to their control littermates ($p<0.0001$, Fig. 5c).

DISCUSSION

This is the first study in a rodent model of enhanced endogenous behavioral despair, ADAR2 transgenic mice where ADAR2 misexpression in the prefrontal cortex results in; 1) perturbed ADAR2 equilibrium, 2) significant changes in editing and altered distribution of edited amino acid isoforms of the $5HT_{2C}R$, 3) no changes in $5HT_{2C}R$, BDNF, CREB, ADAR1 and ADAR3 mRNA expression, 4) no changes in localized levels of biogenic amines levels and 5) reduced basal ERK phosphorylation.

RNA editing of the $5HT_{2C}R$ affects many facets of $5HT_{2C}R$ signaling (Berg *et al.*, 2008, Labasque *et al.*, 2010, Werry *et al.*, 2008a, Werry *et al.*, 2008b) due to the repertoire of edited amino acid isoforms. The five sites that are edited in the pre-mRNA are conserved between human and rodents, but they differ in their levels and patterns of editing (Gardiner & Du, 2006). It is this combinatorial editing at 5 modifiable sites, that results in 32 mRNA variants and 24 amino acid isoforms from a single gene locus.

The significant changes in RNA editing of the $5HT_{2C}R$ in the prefrontal cortex of ADAR2 transgenic mice is most likely a consequence of perturbed ADAR2 equilibrium as expression of other ADARs were unaltered (Fig. 2A) and in addition no changes were detected in local 5-HT levels (Fig. 1b) which have the potential to alter the RNA editing status of the $5HT_{2C}R$ (Gurevich *et al.*, 2002a). At the protein level ADAR2 was found to be only slightly elevated despite the significant increase in total ADAR2 mRNA expression (Fig. 3A, B and C). This could be due to negative feedback loop that regulates the expression of endogenous ADAR2 protein (Feng *et al.*, 2006, Rueter *et al.*, 1999). Therefore the protein expression difference that is observed is probably due to dysregulated rat ADAR2 expression. It is interesting to note this slight change in ADAR2 protein equilibrium coincides with significant RNA editing changes and edited amino acid isoforms of the $5HT_{2C}R$ highlighting the importance of ADAR homeostasis. Further, recent report of increased ADAR1 expression from the cortical region of depressed patients with suicide (Simmons *et al.*, 2010) and reports of altered RNA editing of the $5HT_{2C}R$ in depression (Dracheva *et al.*, 2008b, Gurevich *et al.*, 2002b) highlights the importance of normal levels of ADARs in depression.High throughput pyrosequencing from 466 clones, determined the editing status of $5HT_{2C}R$ mRNA in the prefrontal cortex of control and ADAR2 transgenic mice. ADAR2 transgenic mice showed significant increased editing at A ($P<0.002$), C ($P<0.0009$), D ($P<0.0001$) and E sites ($P<0.042$) compared to their control littermates (Table 2). In rodent model of depression, chronic mouse stress is generally employed to induce depression-like behavior and this kind of manipulation coincides with RNA editing changes in the $5HT_{2C}R$ (Englander *et al.*, 2005, Iwamoto *et al.*, 2005b). A similar observation of increased A, C and E site editing were observed in ADAR2 transgenic mice but without any experimental manipulation. Studies in rodent models of depression and

humans show increased editing at C and E sites reduces the G-protein coupling of the $5HT_{2C}R$ and correlates with depression (Gurevich *et al.*, 2002a, Wang *et al.*, 2000). Thus increased editing C and E sites may have reduced 5-HT signaling in ADAR2 transgenic mice. Since ADAR2 transgenic mice display enhanced endogenous behavior despair, we examined other gene players in depression such as BDNF and CREB as they are influenced by serotonin, stress and antidepressants (Martinowich & Lu, 2008, Qi *et al.*, 2008). There were no changes in BDNF and CREB expression when compared to control littermates thereby suggesting that compromised 5-HT signaling by the modified $5HT_{2C}R$ may be a main contributing factor to affective disorder in ADAR2 mice.

Two members of ADAR family ADAR1 and ADAR2 show preferences for the editing sites in the $5HT_{2C}R$ transcripts with A and B sites preference for ADAR1 and at C and D sites preference by ADAR2 (Beal *et al.*, 2007, Du *et al.*, 2006, Gardiner & Du, 2006, Hartner *et al.*, 2004, Higuchi *et al.*, 2000, Lehmann & Bass, 1999, Lehmann & Bass, 2000, Maas *et al.*, 1999, Wang *et al.*, 2004, Wong *et al.*, 2001), We examined if perturbed ADAR2 equilibrium modified and influenced RNA editing at one site over the other in the context of being either edited or non-edited in ADAR2 transgenic and control mice (Table 4). RNA editing at one site can also disrupt the RNA duplex secondary structure to the extent that ADAR2 cannot properly bind or function on other sites. Overexpression of ADAR2 protein *in vitro* results in competition at the A site editing of ADAR1 (Chen *et al.*, 2000, Singh *et al.*, 2007). Thus, potential for perturbed ADAR2 equilibrium interfering with ADAR1's preferred A and B sites and other site editing were examined. We observed several changes in context of neighboring edited sites influencing the other site editing in ADAR2 transgenic mice; there was a significant increased editing at A site when B site was edited, increased editing at E site when either B or C site were edited and D site increased when C site was edited. However, specificity of D site was lost in ADAR2 transgenic mice. These results suggest certain secondary structures influences the order of editing at the neighboring site and that when perturbed ADAR2 equilibrium is present it leads to promiscuous editing at A, C and E sites resulting in aberrant editing of the $5HT_{2C}R$ in ADAR2 transgenic mice. Similar increased editing changes have been observed in other rodent models of depression (Dracheva *et al.*, 2003, Gurevich *et al.*, 2002a, Gurevich *et al.*, 2002b, Iwamoto & Kato, 2003). It is interesting to note that the recurring theme in all studies show dynamic editing changes at E and C sites in depression and response to drugs and environment stimuli. Further studies are needed to see if behavioral changes correspond to the dynamics of editing in response to environmental stimuli and drug treatments in ADAR2 transgenic mice.

RNA editing at five sites lead to presence of inosines within a short stretch of the transcript that can disrupt the secondary structure of mRNA and destabilize translation. Many inosine can alter base pairing efficiency of tRNAs within the inosine containing codons, lead to nonsense mediated decays and favor nuclear retention of inosine containing RNAs. As a result translational efficiency can be altered (Kozak, 1980, Lim, 1995, Scadden, 2007, Scadden, 2008, Seal *et al.*, 1989). Despite significant increased 3 or more inosines in the $5HT_{2C}R$ transcript of ADAR2 transgenic mice, the total cellular $5HT_{2C}R$ protein expression was unaltered suggesting that translational efficiency of the $5HT_{2C}R$ was unchanged in ADAR2 transgenic mice (Fig. 4a and b and table 3).

Amino acid isoforms of the $5HT_{2C}R$ generated by RNA editing display distinct biochemical and pharmacological properties of the $5HT_{2C}R$ (Berg *et al.*, 2001, Fitzgerald *et al.*, 1999, Labasque *et al.*, 2010, Marion *et al.*, 2004, Mcgrew *et al.*, 2004, Price & Sanders-Bush, 2000, Price *et al.*, 2001, Werry *et al.*, 2008a, Werry *et al.*, 2008b). The general observation is that the unedited INI isoform is the constitutive isoform, has the highest G protein coupling and phospholipase C signaling and that more editing leads to reduced G protein

coupling and phospholipase C signaling (Fig. 6). Modified receptor isoforms have been correlated with behavior (Du *et al.*, 2006, Morabito *et al.*, 2010b) and also differences in editing patterns corresponds to behavioral responses produced by stressful environments (Englander *et al.*, 2005, Gardiner & Du, 2006, Iwamoto *et al.*, 2005b).

We observed significant increase in VNV, VSV, VNI, INV and minor grouped amino acid isoforms and a significant decreased INI amino acid isoform in ADAR2 transgenic mice compared to controls. ERK signaling is coupled to the $5HT_{2C}R$ and editing alters the ERK signaling (Berg *et al.*, 2008, Berg *et al.*, 2001, Labasque *et al.*, 2010, Werry *et al.*, 2008a, Werry *et al.*, 2008b). We therefore examined ERK phosphorylation status in the prefrontal cortex of control and ADAR2 transgenic mice. We observed reduced ERK phosphorylation indicating reduced basal signaling. This observation is consistent with our findings of altered amino acid isoforms of the $5HT_{2C}R$. It is interesting to note that significant reduced constitutive INI isoform coincides with reduced phosphorylation of basal ERK1/2 thus suggesting that 5-HT signaling may be compromised in ADAR2 transgenic mice. However, further studies are required to confirm a direct relationship between pERK1/2 signaling pathways and the edited changes of the $5HT_{2C}R$ and enhanced behavioral despair (Labasque *et al.*, 2010, Singh *et al.*, 2009).

We cannot exclude the possibility that other possible edited isoforms were present but went undetected in our study due to lack of sensitivity by pyrosequencing method and may also play functionally relevant roles in affective disorder. Recently, deep sequencing methods have been used to detect all predicted 24 amino acid isoforms of the $5HT_{2C}R$ (Abbas *et al.*, 2010, Morabito *et al.*, 2010b). Deep sequencing may be useful in the future to detect all the predicted 24 amino acid isoforms in ADAR2 transgenic mice to more thoroughly define which groups of edited isoforms play a role in affective disorder of ADAR2 transgenic mice. Thereafter genetic manipulation to expressing the disease-associated ratio of key edited isoforms in the prefrontal cortex to recapitulate the depression-like behavior of ADAR2 transgenic mice.

Further studies using antidepressants that alter RNA editing of the $5HT_{2C}R$ in ADAR2 transgenic mice will attempt to attenuate the depression-like behavior and will further strengthen the role of edited $5HT_{2C}R$ in affective disorder. Finally we cannot exclude the possibility that other ADAR2 modified substrates may be involved in affective disorder of ADAR2 transgenic mice. Future studies on transcriptome wide analysis using deep sequencing on Solexa platform should identify other substrates with potential contribution to the behavioral phenotypes of ADAR2 transgenic mice. Nevertheless, our findings here suggest that perturbed ADAR2 expression substantially changes the distribution of $5HT_{2C}R$ isoforms which supports the concept that $5HT_{2C}R$ RNA editing is a player in depression.

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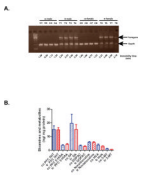

Figure 1. Transgene expression and bioamine levels in the prefrontal cortex of control and ADAR2 transgenic mice

A) RT-PCR analysis on 1.8% agarose gel showing the RT-PCR product of transgene and GAPDH from the prefrontal cortex of control (n=8) and transgenic mice (n=8) from previous study (Singh *et al.*, 2009) that displayed enhanced behavioral despair as determined by their immobility time shown below the gel picture. **B)** Mean ± SEM biogenic amine levels (ng/mg protein) from the prefrontal cortex of control (n=8) and ADAR2 transgenic mice (n=8).

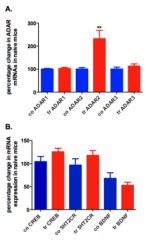

Figure 2. Percentage change in gene expression in the prefrontal cortex of control and ADAR2 transgenic mice

(a) Gene expression of ADAR family in the prefrontal cortex of control and transgenic mice. **(b)** Gene expressions of $5HT_{2C}R$, BDNF and CREB in control (n = 4) and transgenic mice (n = 4) (co, control, tr, transgenic, mean ± SEM, **, $P < 0.001$).

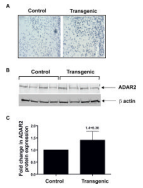

Figure 3. ADAR2 protein expression in the prefrontal cortex of control and transgenic mice

A) A representative picture of immunohistochemistry analysis of ADAR2 expression in the prefrontal cortex of control and transgenic mice. **B)** Western analysis of ADAR2 protein expression from control (n=4) and transgenic mice (n=4). **C)** Histogram showing the fold changes in ADAR2 protein expression relative to control littermates (mean ± SEM 1.4±0.36).

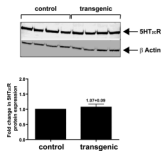

Figure 4. $5HT_{2C}R$ protein expression from the prefrontal cortex of control and ADAR2 transgenic mice

Western analysis of total cellular $5HT_{2C}R$ protein expression in control (n=4) and transgenic mice (n=4) and histogram showing the fold change in $5HT_{2C}R$ protein expression in ADAR2 transgenic mice relative to control littermates (mean±SEM, 1.07±0.09).

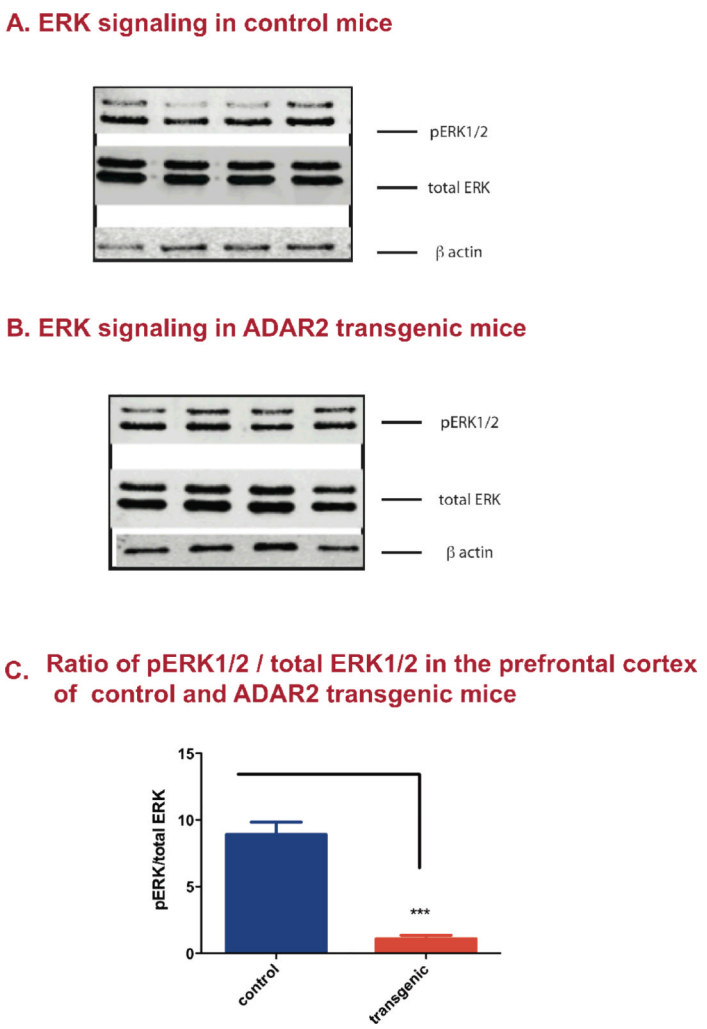

Figure 5. Altered phosphorylated ERK1/2 (pERK1/2) signaling in the prefrontal cortex of control and ADAR2 transgenic mice

Western blot analyses were done on pERK1/2) and total ERK1/2, that were normalized to β actin. To determine the amount of pERK1/2, the ratio of pERK1/2: total ERK1/2 was calculated for each animal and the graph represents the mean ±SEM ratio of pERK1/2: total ERK in control and ADAR2 transgenic mice (n=4/group). **A)** Western blot analysis of pERK1/2, total ERK and β actin protein expression in control mice **B)** Western blot analysis of pERK1/2, total ERK and β actin protein expression in ADAR2 transgenic mice. **C)** Mean ±SEM ratio of pERK1/2/total ERK1/2 in control and ADAR2 transgenic mice is represented the histogram. Student t-test analysis showed that there was a significant decreased basal pERK1/2 in ADAR2 transgenic mice when compared to their control littermates (n=4/ group, mean± SEM, ***=p<0.0001).

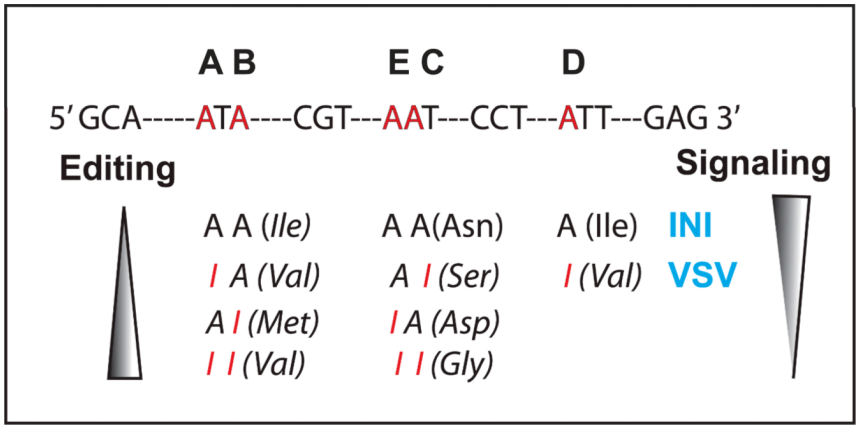

Figure 6. RNA editing and $5HT_{2C}R$ isoforms

Genomic sequence and five modifiable A residues (A-E) in the transcripts of $5HT_{2C}R$ (top), and predicted amino acid changes associated with RNA editing of the A residues in the $5HT_{2C}R$ transcript showing their direction of signaling strength as a function of the amino acid isoforms generated by combinatorial editing (bottom).

Table 1

Primers used for quantitative RT-PCR analysis of gene expression.

Genes	Forward Primer	Reverse Primer
BDNF	5'GCATCTGTTGGGGAGACAAG3'	TGGTCATCACTCTTCTCACCTG3'
ADAR2	5'TCCTGCAGTGACAAGATAGCA3'	5'GGTTCCACGAAAATGCTGAG3'
Transgene	5'GCCCACCACCTACCACGAGTCC3'	5'CGGGGAGTTGGGCCTTGGATCCA3'
ADAR1	5'GAGCAGTTGGGTTTCGCAG3'	5'AAACTGTTGGTCAGAGCATTGAAG3'
ADAR3	5'GGGCATTTCTTCACTTCCTCT3'	5'GGTCCTCTTGATGCTTGCTC3'
CREB	5'CAGGCAGGTGTTTCACAGG	5'GCATGTTCAGAGGGTTAGGG
$5HT_{2c}R$	5'TGCTTAAAACTGAAGCAATAATGG3'	5'AGGCCAATTAGGTGCACAAG3'

Table 2

Quantification of RNA editing at 5 modifiable sites of the $5HT_{2C}R$ in control and transgenic mice

The percentage of editing at each site was calculated from the number of clones sequenced.

Editing site	Probability (95% CI)			Tr vs. Control
	Tr	Control	Odds Ratio (95% CI)	p-value
A	91.0% (86.7%, 94.0%)	82.9% (81.0%, 84.7%)	2.08 (1.32, 3.28)	0.002
B	93.9% (92.0%, 95.3%)	94.6% (91.8%, 96.4%)	0.88 (0.52, 1.48)	0.630
C	20.2% (16.2%, 25.0%)	13.7% (13.3%, 14.1%)	1.60 (1.21, 2.11)	0.0009
D	67.6% (66.0%, 69.0%)	46.5% (45.2%, 47.8%)	2.40 (2.20, 2.62)	0.0001
E	2.8% (1.1%, 7.1%)	0.4% (0.1%, 2.0%)	7.85 (1.08, 57.09)	0.042
No editing	0.5% (0.1%, 3.0%)	5.1% (3.3%, 7.8%)	0.10 (0.02, 0.61)	0.123

Table 3

Number of inosine residues in the $5HT_{2C}R$ transcript of control and transgenic mice

The table represents the proportion of transcripts edited at any combination from 1 to 4 sites. The number of inosine residues was scored from individual clones and the proportion is represented as percentage of total clones sequenced.

Number of Inosine Residue	Probability (95% CI)			Tr vs. Control
	Tr	Control	Odds Ratio (95% CI)	p-value
≥4	15.7% (13.5%, 18.3%)	10.8% (10.2%, 11.4%)	1.54 (1.28, 1.87)	0.0001
≥3	65.7% (65.4%, 66.0%)	49.4% (48.2%, 50.5%)	1.97 (1.88, 2.06)	0.0001

Table 4
RNA editing sites in control and transgenic mice (independence from neighboring sites)

Independence of neighboring sites in transgenic and control mice was tested using the Pearson chi-square test. Independence of neighboring sites in transgenic and control mice was also tested using logistic regression fitted by the GEE method. This analysis was used for all neighboring site pairs except for A and B sites where there were zero cell counts for which computations for fitting a logistic model cannot be performed. For the A-B site pair, Pearson's Chi-square and Cochran-Mantel-Haenszel Chi-square were used if editing at one site influenced the observed frequency of editing at adjacent sites.

Site	Adjacent Site	Probability Site+ (when adjacent site is + or -)		Tr vs. Control p-value
		Tr	Control	
A+	B(+)	97.1%	87.7%	0.0007
	B(-)	0% (n=11)	0% (n=15)	--
	B(+) vs. B(-)	P<0.0001	P<0.0001	
B+	A (+)	100%	100%	--
	A (-)	31.2%	68.1%	0.010
	A (+) vs. A(-)	P<0.0001	P<0.0001	
B+	E(+)	100% (n=5)	100% (n=1)	--
	E(-)	93.8%	94.5%	0.731
	E(+) vs. E(-)	P=1.0	--	
E+	B(+)	2.9%	0.4%	0.038
	B(-)	0% (n=11)	0% (n=15)	--
	B(+) vs. B(-)	P=1.0	P=1.0	
E+	C(+)	8.3%	0%	0.115
	C(-)	1.4%	0.4%	0.560
	C(+) vs. C(-)	P=0.05	P=1.0	
C+	E(+)	60% (n=5)	0% (n=1)	--
	E(-)	18.8%	13.5%	0.134
	C(+) vs. c(-)	P=0.05	--	
C+	D(+)	23.0%	22.6%	0.945
	D(-)	13.6%	6.0%	0.090
	D(+) vs. D(-)	P=0.149	P<0.0001	
D+	C(+)	77.8%	75.7%	0.832
	C(-)	64.8%	40.3%	0.0001
	C(+) VS. C(-)	P=0.149	P<0.0001	

Table 5
Predicted amino acid isoforms of the $5HT_{2c}R$ in control and transgenic mice

cDNAs encoding the predicted amino acid isoforms were scored from individual clones and the frequency of amino acid isoforms were calculated from the total clones sequenced.

Edited Amino Acid Isoform	Probability (95% CI)			Tr vs. Control
	Tr	Control	Odds Ratio	p-value
Valine Asparagine Valine (**Valine Asparagine Valin**)	45.3% (42.2%, 48.4%)	34.5% (32.8%, 36.3%)	1.57 (1.36, 1.82)	0.0001
Valine Serine Valine (**Valin Serine Valin**)	13.3% (12.5%, 14.1%)	10.2% (9.4%, 11.0%)	1.35 (1.20, 1.51)	0.0001
Valine Asparagine Isoleucine (**Valine Asparagine Isoleucine**)	26.0% (23.6%, 28.5%)	34.5% (33.3%, 35.9%)	0.66 (0.58, 0.76)	0.0001
Isoleucine Asparagine Valine (**Isoleucine Asparagine Valine**)	5.0% (4.3%, 5.8%)	0.4% (0.06%, 2.0%)	14.34 (2.53, 81.2)	0.003
Isoleucine Asparagine Isoleucine (**Isoleucine Asparagine Isoleucine**)	0.6% (0.1%, 3.0%)	5.1% (3.4%, 7.6%)	0.10 (0.02, 0.61)	0.012
(Valine Serine Isoleucine) (**VSI**)	3.9% (2.9%, 5.1%)	3.2% (2.6%, 4.0%)	1.19 (0.83, 1.70)	0.342
Valine Aspartic acid Valine (**VDV**), Valine Aspartic acid Isoleucine (**VDI**), Valine Glycine Isoleucine (**VGI**), Valine Glycine Valine (**VGV**), Isoleucine Serine Valine (**ISV**), Methionine Serine Valine (**Msv**), Methionine Asparagine Isoleucine (MNI), Methionine Asparagine Valine (**MNV**)	5.5% (2.1%, 13.9%)	0.4% (0.06%, 2.0%)	16.02 (2.16, 118.6)	0.007