Neurobiology of Disease 39 (2010) 169–180

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

Mice with altered serotonin 2C receptor RNA editing display characteristics of Prader–Willi syndrome

Michael V. Morabito [a,b], Atheir I. Abbas [c], Jennifer L. Hood [a], Robert A. Kesterson [d], Michelle M. Jacobs [a], David S. Kump [d,1], David L. Hachey [b], Bryan L. Roth [e], Ronald B. Emeson [a,b,*]

[a] Center for Molecular Neuroscience, Vanderbilt University, Nashville, TN 37232, USA
[b] Department of Pharmacology, Vanderbilt University, Nashville, TN 37232, USA
[c] Department of Biochemistry, Case Western Reserve University School of Medicine, Cleveland, OH 44106, USA
[d] Department of Genetics, University of Alabama at Birmingham, Birmingham AL 35294, USA
[e] Department of Pharmacology, University of North Carolina, Chapel Hill, NC 27599, USA

ARTICLE INFO

Article history:
Received 23 November 2009
Revised 20 March 2010
Accepted 5 April 2010
Available online 13 April 2010

Keywords:
RNA Editing
Serotonin receptor
Prader–Willi syndrome
Feeding behavior
Metabolism
Hyperphagia
Failure-to-thrive
Hypotonia

ABSTRACT

RNA transcripts encoding the 2C-subtype of serotonin ($5HT_{2C}$) receptor undergo up to five adenosine-to-inosine editing events to encode twenty-four protein isoforms. To examine the effects of altered $5HT_{2C}$ editing *in vivo*, we generated mutant mice solely expressing the fully-edited (VGV) isoform of the receptor. Mutant animals present phenotypic characteristics of Prader–Willi syndrome (PWS) including a failure to thrive, decreased somatic growth, neonatal muscular hypotonia, and reduced food consumption followed by post-weaning hyperphagia. Though previous studies have identified alterations in both $5HT_{2C}$ receptor expression and $5HT_{2C}$-mediated behaviors in both PWS patients and mouse models of this disorder, to our knowledge the $5HT_{2C}$ gene is the first locus outside the PWS imprinted region in which mutations can phenocopy numerous aspects of this syndrome. These results not only strengthen the link between the molecular etiology of PWS and altered $5HT_{2C}$ expression, but also demonstrate the importance of normal patterns of $5HT_{2C}$ RNA editing *in vivo*.

Introduction

The conversion of adenosine to inosine (A-to-I) by RNA editing is a widespread RNA processing event by which genomically encoded sequences are altered through the site-specific deamination of adenosine residue(s) in precursor and mature mRNA transcripts (Gott and Emeson, 2000). The majority of well-characterized A-to-I editing events involve non-synonymous codon changes in RNAs encoding proteins involved in nervous system function including ligand- and voltage-gated ion channels, a G-protein coupled receptor and components of the synaptic release machinery (Burns et al., 1997; Gott and Emeson, 2000; Hoopengardner et al., 2003). Transcripts encoding the $5HT_{2C}$ receptor can be modified by five A-to-I editing events (sites A–E) to generate as many as 24 protein isoforms that differ by up to three amino acids within the predicted second intracellular loop of the receptor, a region involved in receptor:G-protein coupling (Burns et al., 1997; Pin et al., 1994). Though initial sequence analyses of cDNAs isolated from dissected rat and human brains predicted the region-specific expression of as many as 12 major $5HT_{2C}$ receptor isoforms encoded by eighteen distinct RNA species (Burns et al., 1997; Niswender et al., 1999), more recent studies identified the expression of 26 of the 32 possible mRNA isoforms and determined that only 4–6 of these mRNAs represent more than 5% of total $5HT_{2C}$ transcripts in rats and humans, respectively (Dracheva et al., 2009). Alterations in $5HT_{2C}$ receptor editing have been observed in suicide victims with a history of major depression, schizophrenia, or bipolar disorder (Dracheva et al., 2008; Gurevich et al., 2002; Iwamoto and Kato, 2003; Niswender et al., 2001) and in response to antidepressant and antipsychotic treatment (Englander et al., 2005; Gurevich et al., 2002; Sodhi et al., 2005). The fully-edited (VGV) isoform of the human $5HT_{2C}$ receptor, encoding valine, glycine and valine at amino acid positions 156, 158 and 160, respectively, exhibits reduced constitutive activity and decreased G-protein coupling efficacy when compared to the genomically encoded (INI) isoform in heterologous expression systems (Berg et al., 2001; Burns et al., 1997; Fitzgerald et al., 1999; Niswender et al., 1999; Wang et al., 2000), yet the physiologic relevance of $5HT_{2C}$ RNA editing in nervous system function remains unclear.

* Corresponding author. Department of Pharmacology, Vanderbilt University School of Medicine, 465 21st Avenue South, 8140 Medical Research Building 3, Nashville, TN 37232-8548, USA. Fax: +1 615 936 1689.
E-mail address: ron.emeson@vanderbilt.edu (R.B. Emeson).
[1] Present address: Department of Life Sciences, Winston-Salem State University, Winston-Salem, NC 27110, USA.

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doi:10.1016/j.nbd.2010.04.004

Prader–Willi syndrome is a maternally imprinted human disorder resulting from a loss of paternal gene expression on chromosome 15q11–13 that is characterized by a complex phenotype including cognitive deficits, infantile hypotonia and failure to thrive, short stature, hypogonadism and hyperphagia which can lead to morbid obesity (Goldstone, 2004; Nicholls and Knepper, 2001). Multiple mouse models with deficiencies of one or more PWS candidate genes have partially correlated individual genes with aspects of the PWS phenotype (Bischof et al., 2007; Cattanach et al., 1992; Ding et al., 2008; Gabriel et al., 1999; Gerard et al., 1999; Muscatelli et al., 2000; Skryabin et al., 2007; Tsai et al., 1999b; Yang et al., 1998). Among these imprinted candidate genes are the brain-specific small nucleolar RNAs (snoRNAs), HBII-13, HBII-85 and HBII-52 (Cavaille et al., 2000). HBII-52 (*SNORD115*), and its mouse orthologue (MBII-52; *Snord115*), are members of the box C/D family of snoRNAs that are responsible for directing the 2′-*O*-methylation of specific ribose moieties in pre-ribosomal RNA transcripts and U small nuclear RNAs (Kiss, 2002). HBII-52/MBII-52 is complementary to an 18 nucleotide segment of $5HT_{2C}$ mRNA containing three of five editing sites (E, C and D), predicting 2′-*O*-methylation of the ribose for the adenosine nucleoside at the C-site (Cavaille et al., 2000), an editing position that can significantly affect the function of encoded $5HT_{2C}$ protein isoforms (Burns et al., 1997; Niswender et al., 1999). HBII-52/MBII-52 also has been observed to affect $5HT_{2C}$ RNA editing/splicing patterns using tissue culture model systems (Kishore and Stamm, 2006; Vitali et al., 2005). Analyses of $5HT_{2C}$ transcripts have indicated that site-specific editing is increased in brain samples from both PWS patients (Kishore and Stamm, 2006) and a mouse model of PWS (PWS-IC^{del}) that further exhibits deficits in specific $5HT_{2C}$-mediated behaviors (Doe et al., 2009). Though these studies have correlated changes in both $5HT_{2C}$ receptor expression and function in the absence of 15q11–13 gene expression, here we show that increased $5HT_{2C}$ RNA editing in mutant mice recapitulates many aspects of this disorder, suggesting an important role for altered $5HT_{2C}$ function in the etiology of Prader–Willi syndrome.

Materials and methods

Generation of $5HT_{2C\text{-}VGV}$ mice

A genomic fragment containing a portion of the mouse $5HT_{2C}$ gene was isolated from a 129S6 BAC library (Genome Systems, St. Louis, MO) and a 7.4-kb *Avr*II restriction fragment containing exon 5 and a portion of the flanking introns was subcloned into a modified pBKSII$^-$ vector (Stratagene, La Jolla, CA). The five edited adenosine residues within exon 5 were mutated to guanosine moieties using overlap-extension PCR (Ho et al., 1989). A DNA fragment encoding loxP-flanked neomycin phosphotransferase, under control of the phosphoglycerate kinase (PGK) promoter, was inserted into an endogenous *Kpn*I site located 184 bases downstream of the 5′-splice site for exon 5. A selection cassette encoding PGK-driven herpes simplex virus thymidine kinase was inserted outside the region of homology as a negative selectable marker. The *Not*I-linearized targeting vector was introduced into TL-1 embryonic stem cells by electroporation; cells were selected with Geneticin® (G418) and ganciclovir and antibiotic resistant clones were screened for homologous recombination by PCR using the Expand Long Template PCR kit (Roche, USA) using primers located outside the region of homology (sense, 5′-CTGAGTGCATTGGAAAAGAGATCC-3′; antisense, 5′-CCATATATCAGGATGCAGTCTTGTCA-3′). Heterozygous mice obtained from chimeras were mated to mice expressing Cre recombinase under the control of the mouse protamine 1 promoter (The Jackson Laboratory, Bar Harbor, ME) and mutant male offspring were screened for the elimination of the loxP-flanked neomycin resistance cassette. Mutations were verified by DNA sequence analysis and all mice were maintained on the 129S6 strain (Taconic, USA). PCR-based genotyping was employed using primers upstream of the editing sites (5′-AATATCAATAGGTAATTATACC-3′) and downstream of the remaining loxP site within intron 5 (5′-GGGCAAATATTCTGAAAAGATGTT-3′), resulting in the production of 371 and 468 bp amplicons for the wild-type and mutant alleles, respectively. Mutant mice heterozygous for the $5HT_{2C\text{-}VGV}$ allele were mated with wild-type 129S6 animals and subsequent offspring were assessed for the presence of the modified allele and loss of the Cre transgene by segregation.

RNA characterization

Total RNA from male hemizygous $5HT_{2C\text{-}VGV}$ mice and wild-type adult littermates (14 weeks) was isolated from whole brain using Tri-Reagent (Molecular Research Center, Cincinnati, OH) or from dissected brain regions using the PerfectPure RNA tissue kit (5 Prime, Gaithersburg, MD) according to the manufacturer's instructions with all specimens processed in an identical manner. To verify that the introduced A-to-G mutations resulted in the sole production of $5HT_{2C}$ mRNAs encoding the VGV isoform of the receptor, first-strand cDNA was synthesized using AMV reverse transcriptase (Promega, Madison, WI) and amplified using PCR with primers flanking the duplex region (sense primer 5′-AATATCAATAGGTAATTATACC-3′, antisense primer 5′-GGGCAAATATTCTGAAAAGATGTT-3′). Resultant PCR amplicons from both $5HT_{2C\text{-}VGV}$ and wild-type mice were subjected to direct dideoxynucleotide sequence analysis and subsequently subcloned into pBKSII$^-$ (Stratagene, La Jolla, CA) where individual cDNA isolates from each genotype were sequenced.

Quantification of total and alternatively spliced $5HT_{2C}$ mRNAs was performed using either a ribonuclease (RNase) protection assay as described (Emeson et al., 1989), a semi-quantitative RT-PCR strategy, or real-time RT-PCR. For RNase protection analyses, an antisense riboprobe directed against $5HT_{2C}$ pre-mRNA, extending from nucleotide −276 to +316 (relative to the first nucleotide of exon 6), resulted in protected fragments of 602 and 316 nt for pre-mRNA and total $5HT_{2C}$ transcripts, respectively. Three additional antisense riboprobes, specific for alternatively spliced $5HT_{2C}$ RNA isoforms, were developed to unique regions of exon 5 (RNA 1, RNA 2) or the proximal region of intron 5 (RNA 3) and were contiguous with the first 182 nucleotides of exon 6. A cyclophilin antisense probe was also generated as an internal loading control as previously described (nucleotides +34 to +144, GenBank accession number M19533) (Singh et al., 2007). The relative expression of protected fragments was quantified using a Typhoon 9400 phosphorimager (GE Healthcare, Piscataway, NJ) with ImageQuant TL software.

Semi-quantitative analysis of $5HT_{2C}$ mRNA isoform expression by end-point RT-PCR was performed using primers in exons 5 and 6 (sense, 5′-GATATTTGTGCCCCGTC-3′; antisense, 5′-ATCAAAGCTT-GACGGCGTAGGACGTAG-3′) and amplified for 30 cycles. Resultant amplicons were resolved on a 2% agarose gel and ethidium bromide fluorescence was quantified by phosphorimager analysis and values were corrected for the length of each amplicon. Individual $5HT_{2C}$ mRNA isoform expression was determined by summing values corresponding to the RNA1, RNA2, and RNA3 isoforms for each sample and dividing each isoform expression value by this total.

To determine the steady-state mRNA expression levels for $5HT_{2A}$, $5HT_{2B}$, $5HT_{2C}$, and $5HT_7$ receptors by quantitative RT-PCR, first-strand cDNA was synthesized using random hexamers and subjected to Taqman real-time PCR analysis (Applied Biosystems, Foster City, CA). All primers and probes used for the real-time PCR reactions were products of Assay-On-Demand from Applied Biosystems, ($5HT_{2A}$, Assay ID Mm00555764_m1; $5HT_{2B}$, Assay ID Mm00434123_m1; $5HT_{2C}$, Assay ID Mm00434127_m1; $5HT_7$, Assay ID Mm00434133_m1). A probe for Eukaryotic 18S rRNA (product #4319413E, Applied Biosystems) was included in each multiplex PCR reaction as an internal control. Real-time PCR reactions and subsequent analyses were performed with the ABI Prism 7900HT Sequence Detection System (SDS v2.3, Applied Biosystems). To quantify levels of pro-opiomelanocortin (POMC) and neuropeptide Y (NPY), hypothalamic tissue was dissected by the method of Glowinski and Iverson (Glowinski and Iversen, 1966); total