Mice with altered serotonin 2C receptor RNA editing display characteristics of Prader-Willi Syndrome

Michael V. Morabito[1,2], **Atheir I. Abbas**[3], **Jennifer L. Hood**[1], **Robert A. Kesterson**[4], **Michelle M. Jacobs**[1], **David S. Kump**[4,*], **David L. Hachey**[2], **Bryan L. Roth**[5], and **Ronald B. Emeson**[1,2,**]

[1]Center for Molecular Neuroscience, Vanderbilt University, Nashville, TN 37232, USA

[2]Department of Pharmacology, Vanderbilt University, Nashville, TN 37232, USA

[3]Department of Biochemistry, Case Western Reserve University School of Medicine, Cleveland, OH 44106, USA

[4]Department of Genetics, University of Alabama at Birmingham, Birmingham AL 35294, USA

[5]Department of Pharmacology, University of North Carolina, Chapel Hill, NC 27599, USA.

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*.

Keywords

RNA Editing; serotonin receptor; Prader-Willi Syndrome; feeding behavior; metabolism; hyperphagia; failure-to-thrive; hypotonia

---

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.

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 +34-144, 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′-ATCAAAGCTTGACGGCGTAGGACGTAG-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). 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 RNA was isolated and subjected to real-time RT-PCR as described above (NPY, Assay ID Mm00445771_m1; POMC, Assay ID Mm00435874_m1). The amplification efficiencies for all reactions were calculated individually using Real-time PCR Miner software and used to determine the average Ct value for each sample (Zhao and Fernald, 2005). To achieve an accurate quantification of target gene expression, target Ct values were normalized to the corresponding 18S rRNA Ct value for each sample.

Protein expression

Male $5HT_{2C-VGV}$ mice and wild-type littermates were sacrificed at either P2 or 14 weeks of age, whole brains were dissected and immediately frozen on dry ice, followed by manual homogenization in ice-cold sucrose buffer [0.32M sucrose; 10mM K^+-HEPES, pH 7.4, complete EDTA-free protease inhibitor (Roche, Piscataway, NJ)]. After whole cells and nuclei were eliminated by centrifugation at $1000 \times g$ for 10 minutes, microsomes were pelleted at $45000 \times g$ for 15 minutes and resuspended in CHAPS lysis buffer (50mM HEPES, 150mM NaCl, 1mM EDTA, 10mM sodium pyrophosphate, 25mM DTT, 1.5% (w/v) CHAPS, complete EDTA-free protease inhibitor, pH 7.5) by triturating 10x through a 21-gauge needle. Insoluble particles were pelleted at $40000 \times g$ for 20 minutes and the resulting supernatant was incubated overnight with a lectin from *Triticum vulgaris* conjugated to

agarose beads (Sigma-Aldrich, St. Louis, MO) at 4°C. Beads were pelleted at 4000 × g for 1 minute, rinsed with wash buffer (50mM HEPES, 150mM NaCl, 0.1% Triton X-100, 10% glycerol, pH 7.5), and pelleted again. Protein was eluted in 2x SDS sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 200 mM DTT, and 0.2% bromophenol blue) at 65°C for 5 minutes before pelleting the beads and loading the supernatant on a 10% polyacrylamide gel. Following electrophoretic transfer to nitrocellulose, $5HT_{2C}$ protein levels were determined by Western blotting analysis using an anti-$5HT_{2C}$ receptor monoclonal antibody (1:250 [v/v], Santa Cruz Biotechnology, Santa Cruz, CA; SR-2C (D-12)) and an anti-β-actin monoclonal antibody (1:10000 [v/v], Sigma-Aldrich, St. Louis, MO; A1978), respectively, followed by an HRP-labeled anti-mouse secondary antibody (1:1500 [v/v], Vector Labs, Burlingame, CA). The secondary antibody was detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL).

To quantify receptor expression, saturation binding experiments were performed on dissected frontal cortex, striatum, and hippocampus from adult animals. Following anesthesia with isofluorane, mice were cervically dislocated, decapitated, and whole brains were removed from the cranium. A razor blade was used to coronally block the brain immediately anterior to the optic chiasm. The anterior portion of the striatum was identified in the front tissue block. Landmarks used included the white matter separating it from the overlying cortex and the lateral ventricle separating it from the medial wall of the telenchephalon. The caudate-putamen was removed and reserved, with the remainder of the tissue in this block considered frontal cortex. The two hemispheres of the remaining brain tissue were carefully separated from the dorsal surface using forceps to expose the hippocampus and the posterior portion of the striatum. Forceps were used to gently separate both the left and right entire hippocampal structures as a single unit from the rest of the brain, including CA1, CA2, CA3, and dentate gyrus. Following removal of whole hippocampus, the posterior portions of the striatum were visually identified by the striated nature of the tissue and nearby white matter tracts, carefully isolated with forceps, and added to the anterior striatum previously isolated to complete the striatal sample.

Dissected tissue was mechanically homogenized at 15000 rpm for 10 seconds (Tissue Tearor; Biospec Products, Bartlesville, OK) in 2 ml of standard binding buffer (SBB, 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 0.1 mM EDTA) and then centrifuged at 25000 × g for 10 minutes. The supernatant was removed and the pellet was resuspended in 1 ml of SBB and transferred to 1.7 mL eppendorf tubes. The tissue was then spun at 16000 × g in a microcentrifuge for 5 minutes at 4°C. The supernatant was removed and the tissue pellets were frozen at −80°C until used for binding experiments. Total binding was determined using N^6-methyl-$[^3H]$-mesulergine (8-point curves were generated using $[^3H]$-mesulergine concentrations of 0.25, 0.5, 1.0, 1.5, 2.0, 4.0, 6.0, and 8.0 nM) with non-specific binding measured using those same concentrations of $[^3H]$-mesulergine plus 8μM ritanserin; both sets of conditions included 100 nM spiperone to block $5HT_{2A}$ receptor binding. Bradford protein assays were performed to quantify protein in each pellet (BioRad Laboratories, Hercules, CA). All incubations were performed in SBB for 1.5 hours at room temperature. Incubations were terminated by rapid filtration using Whatman GF/C glass fiber filters, washed 3 times with cold 50 mM Tris-HCl (pH 6.9 at room temperature, pH 7.4 at 4°C). Samples were measured by liquid scintillation spectrometry in a Perkin-Elmer Tri-Carb 2800TR counter. Data were analyzed using GraphPad Prism (Graphpad Software, San Diego, CA) using nonlinear regression to fit the experimental data to saturation binding equations (assuming ligand depletion). B_{max} values from Graphpad were normalized to protein concentration as determined using the Bradford assay. Control studies with membranes from $5HT_{2C}$ receptor-null mice (Tecott et al., 1995) demonstrated no appreciable binding (*data not shown*).

Behavioral analysis

Mice were maintained on a 12-h light, 12-h dark cycle in a humidity- and temperature-controlled room with *ad libitum* access to water and standard laboratory chow (rodent chow 5001; Ralston Purina Co., St. Louis, MO). All animal studies were approved by the Institutional Animal Care and Use Committee of Vanderbilt University or the University of Alabama at Birmingham. Growth rates of mutant mice and control littermates were monitored in separate cohorts either daily prior to weaning (P21) or weekly beginning at weaning. Cumulative food consumption was assessed for age-matched, adult $5HT_{2C\text{-}VGV}$ male mice and wild-type littermates using an automated feeding apparatus that measured free-feeding behavior by allowing animals *ad libitum* access to food cups that were mounted on a balance and monitored every 30 seconds (Singh et al., 2007). All feeding studies were performed after animals have been acclimated to the apparatus for at least 72 hrs. Metabolic analyses were assessed for age-matched, adult $5HT_{2C\text{-}VGV}$ male mice and wild-type littermates as previously described (Powell et al., 2002; Singh et al., 2007). Average daily locomotor activity was monitored by both wheel-running activity and infrared motion detection for 21 days, following a 7-day acclimation period and analyzed using ClockLab software (ActiMetrics Software, Wilmette, IL). Acute locomotor activity was measured using open field chambers (Med Associates, St. Albans, VT) and monitored for 60 minutes on 3 successive days as described (Crawley, 2008).

To measure pre-weaning food intake, dams were injected with 6.63mg/g 99.9% deuterium oxide (^2H_2O; Cambridge Isotope Laboratories, Andover, MA) and pups were injected with 3.33mg/g H $^{18}_2$O (Sigma-Aldrich, St. Louis, MO). A graduated glass tuberculin syringe (Becton-Dickson, Franklin Lakes, NJ) used to deliver the isotopic tracers was weighed before and after injection to obtain an accurate mass of the injected dose. Pups were decapitated and blood was harvested at 6, 36, and 72 hours post-injection, with tail-blood simultaneously collected from dams. Samples were centrifuged ($14,000 \times g$) at 4°C for 15 minutes and plasma was analyzed by cavity ring-down infrared spectroscopy to determine the ratios of $^2H_2O/H_2O$ and H_2 $^{18}O/H_2$ ^{16}O (Metabolic Solutions, Nashua, NH) (Crosson et al., 2002). Estimates of water flux in the pups and milk transfer between the dam and pups were made using the methods of Lifson, *et al*. (Lee and Lifson, 1960; Lifson, 1966; McClintock and Lifson, 1957) where the tracer data were expressed as tracer to tracee ratios. A compartmental model was constructed which described the water dilution space and turnover in dams and pups based on the respective dose of ^2H_2O or H_2 ^{18}O. The original single isotope method used in animal studies was modified by incorporation of H_2 $^{18}_2$O to account for independent water turnover in the pups due to metabolic production of water, which leads to about 8% bias, if uncorrected (Butte et al., 1988). SAAM II software (SAAM Institute, University of Washington, Seattle) was used for compartmental analysis (Barrett et al., 1998). Milk intake by each pup was estimated from the rate of transfer of ^2H_2O from the dam to the pup (Infante et al., 1985).

Grip force was measured as described (Meyer et al., 1979) using a force gauge (San Diego Instruments, San Diego, CA). Muscle endurance and motor coordination were assessed using an accelerating Rotarod (Ugo Basile, Comerio, Italy) paradigm. Briefly, mice were placed on a rotating 3.2 cm rod that accelerated from 5-40 revolution/minute over a 5 minute period; latency to fall off of the rod was measured using a stopwatch. Mice that fell from the rod within 15 seconds were retested; mice that grasped the rod and rotated with it for ≥2 revolutions were scored as having fallen.

Statistical analysis

Body weight data were analyzed by two-way analysis of variance (ANOVA). The survival curve was analyzed by the logrank test. Hypothalamic mRNA expression at the P21

timepoint was analyzed by randomized block analysis to eliminate variability in independently collected sample sets. All other data were analyzed by two-tailed Student's *t* test; for data sets with unequal variances, Welch's correction was applied. P values less than 0.05 were considered significant.

Results

Generation of mutant mice solely expressing the fully-edited (VGV) isoform of the $5HT_{2C}$ receptor

To investigate the importance of normal $5HT_{2C}$ RNA editing patterns, we generated mutant mice solely expressing the fully-edited isoform of the receptor ($5HT_{2C\text{-}VGV}$). We chose this receptor isoform based upon its decreased G-protein coupling efficacy and lack of constitutive activity compared to other $5HT_{2C}$ isoforms (Berg et al., 2001; Burns et al., 1997; Fitzgerald et al., 1999; Niswender et al., 1999; Wang et al., 2000). Mice solely expressing $5HT_{2C\text{-}VGV}$ receptors were generated by homologous recombination using a replacement-type targeting vector with the five edited adenosine residues of the $5HT_{2C}$ receptor gene (*htr2C*) mutated to guanosine to mimic the base-pairing properties of inosine (Figs. 1A and B). Since the mouse $5HT_{2C}$ gene is located on the X chromosome, obtaining all possible genotypes of mutant mice and wild-type littermates required two distinct breeding strategies (Table 1). The resultant offspring were genotyped at weaning (postnatal day 21; P21) and demonstrated a normal Mendelian distribution (Fig. 1C and Table 1), indicating that the $5HT_{2C\text{-}VGV}$ mutation does not result in embryonic or early postnatal lethality. To confirm that mutant mice bearing the modified $5HT_{2C\text{-}VGV}$ allele expressed transcripts solely encoding the VGV isoform of the receptor, whole-brain derived cDNA was generated by reverse-transcription polymerase chain reaction (RT-PCR) amplification of $5HT_{2C}$ pre-mRNA and the resulting amplicon was sequenced directly. Complementary DNA from wild-type animals exhibited overlapping adenosine/guanosine peaks in electropherogram traces resulting from a mixture of edited $5HT_{2C}$ transcripts, whereas cDNAs from mutant animals showed only VGV-encoding guanosine moieties at the five editing sites (Fig. 1D). Due to potential alterations in the structure of the extended RNA duplex required for site-selective A-to-I conversion (Burns et al., 1997) resulting from the introduced guanosine mutations at the five editing sites, further sequence analysis of 50 individual cDNA clones derived from $5HT_{2C}$ pre-mRNAs indicated no aberrant editing throughout the predicted region of double-stranded RNA and sole expression of the VGV isoform in $5HT_{2C\text{-}VGV}$ mice (*data not shown*).

Altered $5HT_{2C}$ expression in VGV mutant mice

Co-expression of the HBII-52 snoRNA with $5HT_{2C}$ minigenes has been shown to direct distal 5′-splice site selection in heterologous expression systems through blockade of a splicing silencer that overlaps with the edited region in exon 5 (Kishore and Stamm, 2006; Vitali et al., 2005). Editing of the mutant allele at the E, C, and D sites also weakens this splicing silencer to promote expression of RNA 2 (Fig. 2A) (Flomen et al., 2004), the $5HT_{2C}$ mRNA isoform encoding the full-length receptor protein (Canton et al., 1996; Wang et al., 2000), even in the absence of HBII-52/MBII-52 (Kishore and Stamm, 2006). The other $5HT_{2C}$ splice variants result from the use of a more proximal splice donor in exon 5 (RNA 1) or a distal 5′-splice site in intron 5 (RNA 3), each encoding truncated receptor proteins resulting from premature translation termination that are presumed to be nonfunctional (Fig. 2A) (Canton et al., 1996; Wang et al., 2000). To assess whether the $5HT_{2C\text{-}VGV}$ allele produced alterations in $5HT_{2C}$ mRNA expression, we employed a ribonuclease (RNase) protection strategy using an antisense riboprobe that could distinguish between unspliced premRNA and mature $5HT_{2C}$ transcripts to quantify the level of $5HT_{2C}$ mRNA expression in either whole brain or dissected brain regions (Fig. 2A). Results from

this analysis revealed no detection of pre-mRNA expression nor any difference in total $5HT_{2C}$ mRNA levels (RNA 1 + RNA 2 + RNA 3) for whole brain or dissected brain regions between wild-type and VGV/Y mutant animals (Fig. 2B), although a significant increase and decrease was observed in the levels of RNA 2 and RNA 1, respectively (Figs. 2C and D). Similar results for total $5HT_{2C}$ mRNA expression were observed using a real-time RT-PCR-based strategy (*data not shown*). Since expression of RNA 3 was not detectable using this assay due to its low level of expression, we developed a more sensitive, semi-quantitative RT-PCR-based strategy to amplify the alternatively spliced region for all three $5HT_{2C}$ mRNA isoforms with a single primer pair (Fig. 2D). These results revealed no alteration in RNA 3 expression and confirmed the observed changes in RNA 1 and RNA 2 in mutant mice (Fig. 2C), consistent with previous studies in which increased editing of $5HT_{2C}$ pre-mRNAs promoted the production of RNA 2 at the expense of RNA 1 (Flomen et al., 2004).

While no change in the steady-state level of total $5HT_{2C}$ mRNA was observed in $5HT_{2C}$-VGV mice, mutant animals demonstrated a dramatic increase in receptor protein expression relative to wild-type littermates (40-to-70-fold) that varied between dissected brain regions (Fig. 3). Saturation binding with total membrane fractions isolated from frontal cortex, hippocampus and striatum revealed an increase in maximal binding (B_{max}) with no change in affinity (K_d) for [^{3}H]-mesulergine, a selective 5HT2A/2C antagonist (Fig. 3A). Since $5HT_{2A}$ receptor binding was blocked by the addition of spiperone (100 nM) to all samples, these results indicate a specific increase in $5HT_{2C}$ receptor levels. To determine if a compensatory increase in the expression of other serotonin receptors that can bind [^{3}H]-mesulergine is responsible for the observed increase in binding, real-time RT-PCR analyses were performed to quantify the expression of $5HT_{2A}$, $5HT_{2B}$ and $5HT_7$ receptors in whole brain RNA samples. No significant differences in the steady-state levels of $5HT_{2A}$ and $5HT_7$ receptor transcripts were observed between wild-type and $5HT_{2C-VGV}$ mutant animals, while the expression of $5HT_{2B}$ mRNA was not detectable in whole brain RNA from either genotype (*data not shown*).

We confirmed that the observed differences in $5HT_{2C}$ protein level did not result from *in vivo* differences in pharmacologic properties using a Western blotting strategy from whole brain samples isolated from neonatal (P2) and adult animals (Fig. 3B). Control studies with transiently transfected mouse fibroblasts (NIH-3T3) expressing either $5HT_{2A}$ or $5HT_{2C}$ receptors verified antibody specificity (Fig. 3B). While $5HT_{2C}$ receptor expression was detected readily in protein samples isolated from the whole brain of individual $5HT_{2C-VGV}$ mice at both developmental time points, it was indistinguishable from background in wild-type animals, owing to the low levels of receptor expression observed in many brain regions (Fig. 3A, *right*) (Mengod et al., 1990). These results confirm that the dramatic changes in $5HT_{2C}$ protein expression observed using radio-ligand binding are due to increases in receptor protein expression rather than isoform-specific differences in ligand affinity.

Failure to thrive in 5HT 2C-VGV mutant mice

Initial characterization of $5HT_{2C-VGV}$ mutant animals revealed a 70% decrease in body mass for hemizygous mutant males (VGV/Y) at P21 despite no significant differences in body mass at birth (Fig. 4A), suggesting a postnatal failure to thrive. While a similar reduction in body mass also was observed in homozygous (VGV/VGV) mutant females, heterozygous mutant females (VGV/+) demonstrated an intermediate growth phenotype indicative of haploinsufficiency. Mutant animals appeared hypotonic with reduced milk content in their stomach, subsequently showing clinical signs of dehydration that included piloerection, a mottled appearance and hunched posture (*data not shown*). Mutant mice also demonstrated a post-weaning mortality with 10-20% of mutant offspring dying between P21 and P24 that could be minimized with nutritional supplementation by placing a wet food paste in the

bottom of the cage. In addition to reduced body mass, mutant mice displayed decreased linear growth (Fig. 4A, *inset*), as the body length (nose-to-rump) of VGV/Y males and control littermates (P21) was 60.4 ± 1.3 mm and 87.6 ± 1.5 mm, respectively (n ≥ 5; p ≤ 0.0001). Despite their decreased growth rate prior to P21, all mutant mice bearing the $5HT_{2C\text{-}VGV}$ allele quickly established a normal growth pattern following weaning, gaining mass at a rate roughly parallel to that of their wild-type littermates (Fig. 4B). A gene-dosage effect remained evident in female mice, as homozygous mutants weighed significantly less than heterozygous animals and both groups weighed less than wild-type female littermates (p ≤ 0.0001). This unusual pattern of growth, switching from an initial failure to thrive to a post-weaning weight gain that parallels wild-type littermates, is similar to that previously observed for a mouse model of PWS (PWS-IC^{del}) in which a 42 kilobase (kb) region was deleted from the distal portion of the putative mouse imprinting center on chromosome 7 (Chamberlain et al., 2004; Yang et al., 1998).

Altered feeding behavior and increased metabolism in $5HT_{2C\text{-}VGV}$ mice

Infants with PWS initially present with feeding difficulties and a failure to thrive, often requiring gavage or other medical intervention to maintain proper nutrition (Goldstone, 2004). To determine if the observed failure to thrive in $5HT_{2C\text{-}VGV}$ mice resulted from similar feeding deficits, we measured milk consumption in mice from postnatal day 9-12. Offspring derived from a wild-type male x heterozygous mutant female breeding strategy were genotyped at P8 and the individual litters were culled to 3 pups at P9, with one pup of each possible genotype represented in each litter (wild-type, VGV/+, and VGV/Y). Pups and dams were injected with separate, stable heavy water isotopes (H_2 ^{18}O and ^2H_2O, respectively) on P9 and the accumulation and clearance of the isotopes was measured at regular intervals over a 72 hour period. $5HT_{2C\text{-}VGV}$ mice ingested significantly less milk than wild-type littermates (0.580 ± 0.097 and 1.345 ± 0.301 grams milk/day, respectively; p < 0.05), with heterozygous mutant animals again exhibiting an intermediate phenotype (1.092 ± 0.212 grams milk/day), suggesting that decreased pre-weaning milk consumption contributes to the reduced growth rate of mutant mice.

The $5HT_{2C}$ receptor has been implicated directly in the anorectic modulation of food intake using both transgenic and pharmacologic approaches (Heisler et al., 2002; Hewitt et al., 2002; Nonogaki et al., 1998; Tecott et al., 1995). Despite the 22% reduction in the mean body mass for adult $5HT_{2C\text{-}VGV}$ mice compared to their wild-type littermates (Fig. 4B), age-matched adult mutant and wild-type animals consume roughly equivalent amounts of food (*data not shown*). Since adult animals of even slightly different weights would be expected to demonstrate differences in food consumption due to the dominance of anabolic pathways to increase feeding to maintain adipose mass (Augustine and Rossi, 1999), food intake was corrected for body mass to reveal that $5HT_{2C\text{-}VGV}$ mice were functionally hyperphagic with an 18% increase in daily food consumption (Fig. 5A).

Individuals with PWS classically present with hyperphagia and impaired satiety, often leading to severe obesity in affected patients as young as two years of age (Goldstone, 2004). Despite their hyperphagia, $5HT_{2C\text{-}VGV}$ mice do not become obese and maintain the 7-10 gram reduction in body mass compared to wild-type littermates initially observed at weaning (Fig. 4B). This absence of obesity was not associated with a corresponding increase in locomotor activity, as no significant differences were observed between $5HT_{2C\text{-}VGV}$ and wild-type animals when assessed by home cage analysis of infrared motion detection (Fig. 5B) or wheel running activity (*data not shown*). However, indirect calorimetry revealed that $5HT_{2C\text{-}VGV}$ mice expend more total and resting energy than wild-type littermates per gram body mass (Fig. 5C), suggesting that an elevated metabolic rate in mutant animals may compensate for hyperphagia to prevent the subsequent onset of obesity.

Perceived energy deficits increase expression of the orexigenic neuropeptide Y (NPY) (Mizuno et al., 1999) while simultaneously decreasing the expression of anorectic peptides derived from the pro-opiomelanocortin (POMC) prohormone (Schwartz et al., 1997) to increase food consumption. Quantitative analysis of hypothalamic RNA expression in mutant animals revealed a 22-89% increase in NPY mRNA relative to wild-type mice at all time points examined, whereas steady-state POMC mRNA levels were significantly decreased 76% at weaning (P21), 29% at 14 weeks and 33% at 36 weeks of age, but were unaltered at P4 (Fig. 5D). These changes in hypothalamic neuropeptide mRNA expression are consistent with the observed post-weaning increase in food consumption in $5HT_{2C\text{-}VGV}$ animals (Ellacott and Cone, 2004).

$5HT_{2C\text{-}VGV}$ mice display additional PWS characteristics observed in humans or mouse models

Infants affected with PWS exhibit other nonspecific symptoms including hypotonia and growth retardation (Goldstone, 2004), with similar phenotypes also observed in several mouse models of PWS (Ding et al., 2008; Gabriel et al., 1999; Skryabin et al., 2007; Tsai et al., 1999b; Yang et al., 1998). To examine potential changes in muscle tone, we initially employed an accelerating Rotarod® paradigm. Recently weaned wild-type animals outperformed their mutant littermates on this task, with no difference observed in adults (Fig. 6A). Since decreased performance using the Rotarod® could reflect alterations in either muscle tone or motor coordination, we also measured the grip strength of wild-type and hemizygous male $5HT_{2C\text{-}VGV}$ mice (Fig. 6B). Though no differences were observed in adult mice, weanling mutant animals (P21-P28) exhibited a ~50% reduction in grip force, suggesting that an early hypotonia in $5HT_{2C\text{-}VGV}$ mice improves with age similar to patients affected with PWS (Goldstone, 2004).

Several previously described murine models of PWS report feeding difficulties, failure to thrive and/or growth deficiency, yet the utility of these models has been hampered by substantial neonatal lethality (Cattanach et al., 1992; Gerard et al., 1999; Tsai et al., 1999b; Yang et al., 1998). More recent studies have identified permissive (FVB/NJ, C3H/HeJ, 129S1/Sv and BALB/cJ) and non-permissive (C57BL/6J and DBA/2J) mouse background strains that either allow survival or result in neonatal mortality, respectively, when bearing deletions within the PWS critical region on chromosome 7C (Chamberlain et al., 2004; Gerard et al., 1999). To compare background-dependent phenotypic differences between $5HT_{2C\text{-}VGV}$ mutant mice and other PWS models, 129S6-derived heterozygous $5HT_{2C\text{-}VGV}$ dams were mated with wild-type C57BL/6J males. Offspring were genotyped at weaning or at the time of death to reveal a completely penetrant neonatal lethality for hemizygous mutant males, with 75% dying between P8-P12 and none surviving beyond P15 (Fig. 7; $p \leq 0.0001$). These results are similar to the early neonatal lethality observed for other mouse models of PWS on the C57BL/6 background (Chamberlain et al., 2004; Gerard et al., 1999) and further indicate that while a single mutant $5HT_{2C}$ allele can cause alterations in growth and receptor expression in both hemizygous males and heterozygous females (Fig. 4) (U. B. Olaghere da Silva, 2009), the presence of a single wild-type allele is sufficient for survival on a non-permissive background strain (Fig. 7).

Discussion

The editing of $5HT_{2C}$ transcripts modulates multiple aspects of $5HT_{2C}$ receptor signaling and expression in heterologous systems (Berg et al., 2001; Burns et al., 1997; Fitzgerald et al., 1999; Flomen et al., 2004; Marion et al., 2004; Niswender et al., 1999; Price et al., 2001; Wang et al., 2000), yet the physiologic importance for the existence of multiple $5HT_{2C}$ isoforms has not been fully explored. Recent studies by Kawahara *et al*. have characterized mutant mice comparable to those described in the present study in which $5HT_{2C}$ expression

was limited to the fully-edited (VGV) isoform. These animals exhibited several similar phenotypic alterations, including reduced pre-weaning growth, post-weaning hyperphagia and an increased metabolic rate resulting from excessive activation of the sympathetic nervous system (Kawahara et al., 2008), yet several differences between these mutant lines also were readily apparent. Mice solely expressing the VGV isoform were previously reported to exhibit reduced locomotor activity in an open field chamber (Kawahara et al., 2008). While similar results were observed with $5HT_{2C\text{-}VGV}$ mice using such a behavioral paradigm (*data not shown*), these animals exhibited normal activity in their home cage whether quantified by infrared motion detection (Fig. 5B) or wheel running (*data not shown*). This suggests that mice solely expressing the fully-edited $5HT_{2C}$ isoform do not exhibit baseline hypoactivity, but rather decreased activity in a novel environment. Unlike the dramatic increase in $5HT_{2C}$ receptor expression observed in $5HT_{2C\text{-}VGV}$ animals using both radioligand binding (Fig. 3A) and Western blotting (Fig. 3B) analyses, the mutant animals described by Kawahara *et al.* did not exhibit any increase in $5HT_{2C}$ receptor protein when analyzed by Western blotting (Kawahara et al., 2008). This result is particularly surprising given that $5HT_{2C}$-selective autoradiographic analyses by Kawahara *et al*. revealed an increase in receptor expression that was inconsistent with the Western blotting studies for these animals (Kawahara et al., 2008).

The 40-to-70-fold increase in total $5HT_{2C}$ receptor protein observed in $5HT_{2C\text{-}VGV}$ mutant animals (Fig. 3) was unexpected, given the minor nature of the mutations introduced into exon 5 of the endogenous *htr2C* locus (Figs. 1A and B) and the fact that there were no observed alterations in the steady-state levels of $5HT_{2C}$ transcripts (Fig. 6*B*). Although a 27% increase in the production of RNA 2 was observed in mutant mice (Figs. 2C and D), the dramatic increase in functional $5HT_{2C}$ protein levels cannot be explained readily by this small alteration in $5HT_{2C}$ splice-site selection. Increased receptor protein could represent an adaptive mechanism to compensate for the diminished coupling efficacy and constitutive activity of the $5HT_{2C\text{-}VGV}$ isoform that was observed initially in transfected cell lines (Burns et al., 1997;Fitzgerald et al., 1999;Niswender et al., 1999;Wang et al., 2000). The absence of a change in total $5HT_{2C}$ transcripts for mutant animals indicates the existence of previously unrecognized post-transcriptional mechanism(s) to regulate $5HT_{2C}$ receptor density that could be mediated by potential differences in translation efficiency and/or stability for distinct edited receptor isoforms. Recent studies by Marion *et al*. have provided a potential mechanism for isoform-specific differences in protein stability based upon a constitutive activity-dependent alteration in the subcellular compartmentalization of the receptor, resulting from an inability of highly edited $5HT_{2C}$ receptor isoforms to effectively interact with β-arrestin2 (Marion et al., 2004).

While the hyperphagia observed in $5HT_{2C\text{-}VGV}$ mutant mice is similar to that exhibited by $5HT_{2C}$ receptor-null animals, null mutants develop adult-onset obesity with no change in metabolic rate (Nonogaki et al., 2003; Nonogaki et al., 1998); however, $5HT_{2C\text{-}VGV}$ mutant mice display an increase in their resting energy expenditure in the absence of hyperactivity, which likely compensates for the hyperphagia observed in these mutants to prevent the onset of obesity. The orexigenic changes in hypothalamic POMC and NPY mRNA expression in $5HT_{2C\text{-}VGV}$ mutant animals are consistent with the observed increase in relative food consumption (Fig. 5A). These data support previous evidence for a functional role for $5HT_{2C}$ receptors in the modulation of food intake via a POMC-mediated mechanism, including the expression of $5HT_{2C}$ transcripts in ~80% of POMC neurons within the arcuate nucleus of the hypothalamus (Heisler et al., 2002) and recent studies demonstrating that the selective ablation of $5HT_{2C}$ in these neurons is sufficient to eliminate the effects of serotonergic compounds on food intake and result in hyperphagia-mediated obesity (Xu et al., 2008).

Although multiple mouse models lacking expression of one or more PWS candidate genes have implicated several loci with elements of the PWS phenotype, these models vary widely in their capacity to recapitulate the human disorder (Table 2). Mouse models lacking expression of individual genes located within the imprinted region on chromosome 7C display either no obvious PWS characteristics (Jong et al., 1999;Tsai et al., 1999a;Tsai et al., 1999b;Yang et al., 1998), neonatal lethality from respiratory distress (Gerard et al., 1999), or body mass changes that never differ from wild-type animals by more than 10% (Bischof et al., 2007). However, several functional deletions encompassing larger portions of the imprinted region result in neonatal lethality preceded by a failure to thrive (Cattanach et al., 1992;Chamberlain et al., 2004;Gerard et al., 1999;Tsai et al., 1999b;Yang et al., 1998). It is important to note that none of the mouse models of PWS reported to date demonstrate either the infertility or the morbid obesity characteristic of the human syndrome, suggesting that mice may not be capable of manifesting the full spectrum of phenotypic changes associated with this genetic disorder. Reasons for the limited PWS phenotype(s) observed in mice may include species-specific differences in gene structure, function, cell-expression patterns, neuroendocrine and metabolic pathways and/or modifier genes (Goldstone, 2004).

Mutant mice solely expressing the fully-edited isoform of the $5HT_{2C}$ receptor display several phenotypic characteristics of PWS, including a failure to thrive, decreased somatic growth and neonatal muscular hypotonia, followed by post-weaning hyperphagia, in addition to other similarities with mouse models of PWS where deletions within the imprinting center on chromosome 7C result in strain-specific neonatal lethality. These observations are consistent with recent analyses indicating that $5HT_{2C}$ RNA editing is increased in autopsy samples from PWS patients (Kishore and Stamm, 2006) and a mouse model (PWS-IC[del]) that also demonstrates alterations in $5HT_{2C}$-related behaviors (Doe et al., 2009). Previous studies have shown that the maternally imprinted snoRNA, HBII-52, can alter both the splicing and editing of $5HT_{2C}$ transcripts (Kishore and Stamm, 2006), providing a provoking and straightforward mechanism by which to link the $5HT_{2C\text{-}VGV}$ mutation with the 15q11-13 locus and multiple aspects of this disorder. However, several clinical cases have been identified recently that either suggest deletion of this snoRNA is not sufficient to cause a PWS-like phenotype (Runte et al., 2005) or that a specific paternal deletion of the HBII-85 snoRNA cluster can result in PWS (de Smith et al., 2009; Gallagher et al., 2002; Sahoo et al., 2008). Due to the nature of these human clinical studies however, it was impossible to assess potential changes in the brain-specific expression of other snoRNAs in these patients, negating the ability of such studies to conclusively eliminate a role for HBII-52. The generation of mutant mice bearing similar deletions have produced animals with multiple PWS-like phenotypes (Table 2) and have shown the expression of MBII-52 snoRNAs in the brains of these mutant animals, suggesting that the absence of MBII-52 alone is not sufficient to cause this syndrome (Ding et al., 2008; Ding et al., 2005; Skryabin et al., 2007). While most recent clinical and mouse studies have focused upon the HBII-85 snoRNA cluster as playing a causal role in PWS (de Smith et al., 2009; Ding et al., 2008; Gallagher et al., 2002; Runte et al., 2005; Skryabin et al., 2007), the targets of these imprinted snoRNAs remain largely unknown, raising the possibility that alternative mechanisms exist by which genes located within the 15q11-13 locus can alter $5HT_{2C}$ receptor function independent of HBII-52 expression.

The present studies have demonstrated that alterations in the normal pattern(s) of editing can result in significant changes in growth, metabolism and feeding behavior and also have identified an unknown post-transcriptional mechanism to modulate $5HT_{2C}$ receptor density. Given the ability of the $5HT_{2C\text{-}VGV}$ mutation to phenocopy the failure-to-thrive, reduced early-postnatal growth, hypotonia, adult hyperphagia, and strain-specific lethality observed in previously-described mouse models of PWS (Table 2), these results strongly suggest a link between altered $5HT_{2C}$ receptor function and the development of major criteria

necessary for the diagnosis of this disorder (Gunay-Aygun et al., 2001). Though this model may implicate increased $5HT_{2C}$ RNA editing in the molecular etiology of PWS, it does not suggest that the full spectrum of altered phenotypes observed in PWS patients result solely from altered $5HT_{2C}$ receptor function. When combined with previously identified alterations in $5HT_{2C}$-mediated behaviors in PWS-IC[del] mice (Doe et al., 2009), observed increases in the editing of $5HT_{2C}$ transcripts in both mouse model of PWS and human patients (Doe et al., 2009;Kishore and Stamm, 2006), and the known involvement of the $5HT_{2C}$ receptor in controlling energy homeostasis and satiety (Hewitt et al., 2002;Somerville et al., 2007;Xu et al., 2008), the presence of PWS-like phenotypes in mice with altered $5HT_{2C}$ editing identifies the *htr2C* gene as the first locus outside the 15q11-13 imprinted region in which mutations can recapitulate multiple aspects of this human genetic disorder.

Acknowledgments

References

Augustine KA, Rossi RM. Rodent mutant models of obesity and their correlations to human obesity. Anat Rec 1999;257:64–72. [PubMed: 10321434]

Barrett PH, et al. SAAM II: Simulation, Analysis, and Modeling Software for tracer and pharmacokinetic studies. Metabolism 1998;47:484–92. [PubMed: 9550550]

Berg KA, et al. RNA-editing of the 5-HT(2C) receptor alters agonist-receptor-effector coupling specificity. Br J Pharmacol 2001;134:386–92. [PubMed: 11564657]

Bischof JM, et al. Inactivation of the mouse Magel2 gene results in growth abnormalities similar to Prader-Willi syndrome. Hum Mol Genet 2007;16:2713–9. [PubMed: 17728320]

Burns CM, et al. Regulation of serotonin-2C receptor G-protein coupling by RNA editing. Nature 1997;387:303–8. [PubMed: 9153397]

Butte NF, et al. Human-milk intake measured by administration of deuterium oxide to the mother: a comparison with the test-weighing technique. Am J Clin Nutr 1988;47:815–21. [PubMed: 2834941]

Canton H, et al. Identification, molecular cloning, and distribution of a short variant of the 5-hydroxytryptamine2C receptor produced by alternative splicing. Mol Pharmacol 1996;50:799–807. [PubMed: 8863824]

Cattanach BM, et al. A candidate mouse model for Prader-Willi syndrome which shows an absence of Snrpn expression. Nat Genet 1992;2:270–4. [PubMed: 1303278]

Cavaille J, et al. Identification of brain-specific and imprinted small nucleolar RNA genes exhibiting an unusual genomic organization. Proc Natl Acad Sci U S A 2000;97:14311–6. [PubMed: 11106375]

Chamberlain SJ, et al. Evidence for genetic modifiers of postnatal lethality in PWS-IC deletion mice. Hum Mol Genet 2004;13:2971–7. [PubMed: 15459179]

Crawley JN. Behavioral phenotyping strategies for mutant mice. Neuron 2008;57:809–18. [PubMed: 18367082]

Crosson ER, et al. Stable isotope ratios using cavity ring-down spectroscopy: determination of 13C/ 12C for carbon dioxide in human breath. Anal Chem 2002;74:2003–7. [PubMed: 12033299]

de Smith AJ, et al. A Deletion of the HBII-85 Class of Small Nucleolar RNAs (snoRNAs) is Associated with Hyperphagia, Obesity and Hypogonadism. Hum Mol Genet. 2009

Ding F, et al. SnoRNA Snord116 (Pwcr1/MBII-85) deletion causes growth deficiency and hyperphagia in mice. PLoS ONE 2008;3:e1709. [PubMed: 18320030]

Ding F, et al. Lack of Pwcr1/MBII-85 snoRNA is critical for neonatal lethality in Prader-Willi syndrome mouse models. Mamm Genome 2005;16:424–31. [PubMed: 16075369]

Doe CM, et al. Loss of the imprinted snoRNA mbii-52 leads to increased 5htr2c pre-RNA editing and altered 5HT2CR-mediated behaviour. Hum Mol Genet 2009;18:2140–8. [PubMed: 19304781]

Dracheva S, et al. Editing of serotonin 2C receptor mRNA in the prefrontal cortex characterizes high-novelty locomotor response behavioral trait. Neuropsychopharmacology 2009;34:2237–51. [PubMed: 19494808]

Dracheva S, et al. Increased serotonin 2C receptor mRNA editing: a possible risk factor for suicide. Mol Psychiatry 2008;13:1001–10. [PubMed: 17848916]

Ellacott KL, Cone RD. The central melanocortin system and the integration of short- and long-term regulators of energy homeostasis. Recent Prog Horm Res 2004;59:395–408. [PubMed: 14749511]

Emeson RB, et al. Alternative production of calcitonin and CGRP mRNA is regulated at the calcitonin-specific splice acceptor. Nature 1989;341:76–80. [PubMed: 2788825]

Englander MT, et al. How stress and fluoxetine modulate serotonin 2C receptor pre-mRNA editing. J Neurosci 2005;25:648–51. [PubMed: 15659601]

Fitzgerald LW, et al. Messenger RNA editing of the human serotonin 5-HT2C receptor. Neuropsychopharmacology 1999;21:82S–90S. [PubMed: 10432493]

Flomen R, et al. Evidence that RNA editing modulates splice site selection in the 5-HT2C receptor gene. Nucleic Acids Res 2004;32:2113–22. [PubMed: 15087490]

Gabriel JM, et al. A transgene insertion creating a heritable chromosome deletion mouse model of Prader-Willi and angelman syndromes. Proc Natl Acad Sci U S A 1999;96:9258–63. [PubMed: 10430930]

Gallagher RC, et al. Evidence for the role of PWCR1/HBII-85 C/D box small nucleolar RNAs in Prader-Willi syndrome. Am J Hum Genet 2002;71:669–78. [PubMed: 12154412]

Gerard M, et al. Disruption of the mouse necdin gene results in early post-natal lethality. Nat Genet 1999;23:199–202. [PubMed: 10508517]

Glowinski J, Iversen LL. Regional studies of catecholamines in the rat brain. I. The disposition of [3H]norepinephrine, [3H]dopamine and [3H]dopa in various regions of the brain. J Neurochem 1966;13:655–69. [PubMed: 5950056]

Goldstone AP. Prader-Willi syndrome: advances in genetics, pathophysiology and treatment. Trends Endocrinol Metab 2004;15:12–20. [PubMed: 14693421]

Gott JM, Emeson RB. Functions and mechanisms of RNA editing. Annu Rev Genet 2000;34:499–531. [PubMed: 11092837]

Gunay-Aygun M, et al. The changing purpose of Prader-Willi syndrome clinical diagnostic criteria and proposed revised criteria. Pediatrics 2001;108:E92. [PubMed: 11694676]

Gurevich I, et al. Modulation of serotonin 2C receptor editing by sustained changes in serotonergic neurotransmission. J Neurosci 2002;22:10529–32. [PubMed: 12486144]

Heisler LK, et al. Activation of central melanocortin pathways by fenfluramine. Science 2002;297:609–11. [PubMed: 12142539]

Hewitt KN, et al. Serotonin 2C receptor agonists and the behavioural satiety sequence in mice. Pharmacol Biochem Behav 2002;71:691–700. [PubMed: 11888560]

Ho SN, et al. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene 1989;77:51–9. [PubMed: 2744487]

Hoopengardner B, et al. Nervous system targets of RNA editing identified by comparative genomics. Science 2003;301:832–6. [PubMed: 12907802]

Infante C, et al. Isotope dilution measurement of breast-milk production in Chilean urban mothers. Hum Nutr Clin Nutr 1985;39:379–86. [PubMed: 4055429]

Iwamoto K, Kato T. RNA editing of serotonin 2C receptor in human postmortem brains of major mental disorders. Neurosci Lett 2003;346:169–72. [PubMed: 12853111]

Jong MT, et al. Imprinting of a RING zinc-finger encoding gene in the mouse chromosome region homologous to the Prader-Willi syndrome genetic region. Hum Mol Genet 1999;8:795–803. [PubMed: 10196368]

Kawahara Y, et al. Dysregulated editing of serotonin 2C receptor mRNAs results in energy dissipation and loss of fat mass. J Neurosci 2008;28:12834–44. [PubMed: 19036977]

Kishore S, Stamm S. The snoRNA HBII-52 regulates alternative splicing of the serotonin receptor 2C. Science 2006;311:230–2. [PubMed: 16357227]

Kiss T. Small nucleolar RNAs: an abundant group of noncoding RNAs with diverse cellular functions. Cell 2002;109:145–8. [PubMed: 12007400]

Lee JS, Lifson N. Measurement of total energy and material balance in rats by means of doubly labeled water. Am J Physiol 1960;199:238–42. [PubMed: 13760247]

Lifson N. Theory of use of the turnover rates of body water for measuring energy and material balance. J Theor Biol 1966;12:46–74. [PubMed: 6015431]

Marion S, et al. RNA editing induces variation in desensitization and trafficking of 5-hydroxytryptamine 2c receptor isoforms. J Biol Chem 2004;279:2945–54. [PubMed: 14602721]

McClintock R, Lifson N. Applicability of the D2O18 method to the measurement of the total carbon dioxide output of obese mice. J Biol Chem 1957;226:153–6. [PubMed: 13428747]

Mengod G, et al. The distribution and cellular localization of the serotonin 1C receptor mRNA in the rodent brain examined by in situ hybridization histochemistry. Comparison with receptor binding distribution. Neuroscience 1990;35:577–91. [PubMed: 2381516]

Meyer OA, et al. A method for the routine assessment of fore- and hindlimb grip strength of rats and mice. Neurobehav Toxicol 1979;1:233–6. [PubMed: 551317]

Mizuno TM, et al. Fasting regulates hypothalamic neuropeptide Y, agouti-related peptide, and proopiomelanocortin in diabetic mice independent of changes in leptin or insulin. Endocrinology 1999;140:4551–7. [PubMed: 10499510]

Muscatelli F, et al. Disruption of the mouse Necdin gene results in hypothalamic and behavioral alterations reminiscent of the human Prader-Willi syndrome. Hum Mol Genet 2000;9:3101–10. [PubMed: 11115855]

Nicholls RD, Knepper JL. Genome organization, function, and imprinting in Prader-Willi and Angelman syndromes. Annu Rev Genomics Hum Genet 2001;2:153–75. [PubMed: 11701647]

Niswender CM, et al. RNA editing of the human serotonin 5-hydroxytryptamine 2C receptor silences constitutive activity. J Biol Chem 1999;274:9472–8. [PubMed: 10092629]

Niswender CM, et al. RNA editing of the human serotonin 5-HT2C receptor. alterations in suicide and implications for serotonergic pharmacotherapy. Neuropsychopharmacology 2001;24:478–91. [PubMed: 11282248]

Nonogaki K, et al. Hyperactivity and reduced energy cost of physical activity in serotonin 5-HT(2C) receptor mutant mice. Diabetes 2003;52:315–20. [PubMed: 12540602]

Nonogaki K, et al. Leptin-independent hyperphagia and type 2 diabetes in mice with a mutated serotonin 5-HT2C receptor gene. Nat Med 1998;4:1152–6. [PubMed: 9771748]

Pin JP, et al. Domains involved in the specificity of G protein activation in phospholipase C-coupled metabotropic glutamate receptors. EMBO J 1994;13:342–8. [PubMed: 8313879]

Powell CS, et al. Effects of energy expenditure and Ucp1 on photoperiod-induced weight gain in collard lemmings. Obes Res 2002;10:541–50. [PubMed: 12075603]

Price RD, et al. RNA editing of the human serotonin 5-HT2C receptor alters receptor-mediated activation of G13 protein. J Biol Chem 2001;276:44663–8. [PubMed: 11572865]

Runte M, et al. Exclusion of the C/D box snoRNA gene cluster HBII-52 from a major role in Prader-Willi syndrome. Hum Genet 2005;116:228–30. [PubMed: 15565282]

Sahoo T, et al. Prader-Willi phenotype caused by paternal deficiency for the HBII-85 C/D box small nucleolar RNA cluster. Nat Genet 2008;40:719–21. [PubMed: 18500341]

Schwartz MW, et al. Leptin increases hypothalamic pro-opiomelanocortin mRNA expression in the rostral arcuate nucleus. Diabetes 1997;46:2119–23. [PubMed: 9392508]

Singh M, et al. Hyperphagia-mediated obesity in transgenic mice misexpressing the RNA-editing enzyme ADAR2. J Biol Chem 2007;282:22448–59. [PubMed: 17567573]

Skryabin BV, et al. Deletion of the MBII-85 snoRNA gene cluster in mice results in postnatal growth retardation. PLoS Genet 2007;3:e235. [PubMed: 18166085]

Sodhi MS, et al. A rapid new assay to detect RNA editing reveals antipsychotic-induced changes in serotonin-2C transcripts. Mol Pharmacol 2005;68:711–9. [PubMed: 15917433]

Somerville EM, et al. 5-HT(2C) receptor activation inhibits appetitive and consummatory components of feeding and increases brain c-fos immunoreactivity in mice. Eur J Neurosci 2007;25:3115–24. [PubMed: 17561825]

Tecott LH, et al. Eating disorder and epilepsy in mice lacking 5-HT2c serotonin receptors. Nature 1995;374:542–6. [PubMed: 7700379]

Tsai TF, et al. Necdin-deficient mice do not show lethality or the obesity and infertility of Prader-Willi syndrome. Nat Genet 1999a;22:15–6. [PubMed: 10319852]

Tsai TF, et al. Paternal deletion from Snrpn to Ube3a in the mouse causes hypotonia, growth retardation and partial lethality and provides evidence for a gene contributing to Prader-Willi syndrome. Hum Mol Genet 1999b;8:1357–64. [PubMed: 10400982]

Olaghere da Silva UB, C. CE, Morabito MV, Jacobs MM, Emeson RB, Li D, Airey DC, Sanders-Bush E. Impact of RNA editing on functions of the serotonin 2C receptor *in vivo*. 2009 in submission.

Vitali P, et al. ADAR2-mediated editing of RNA substrates in the nucleolus is inhibited by C/D small nucleolar RNAs. J Cell Biol 2005;169:745–53. [PubMed: 15939761]

Wang Q, et al. Altered G protein-coupling functions of RNA editing isoform and splicing variant serotonin2C receptors. J Neurochem 2000;74:1290–300. [PubMed: 10693963]

Xu Y, et al. 5-HT2CRs expressed by pro-opiomelanocortin neurons regulate energy homeostasis. Neuron 2008;60:582–9. [PubMed: 19038216]

Yang T, et al. A mouse model for Prader-Willi syndrome imprinting-centre mutations. Nat Genet 1998;19:25–31. [PubMed: 9590284]

Zhao S, Fernald RD. Comprehensive algorithm for quantitative real-time polymerase chain reaction. J Comput Biol 2005;12:1047–64. [PubMed: 16241897]
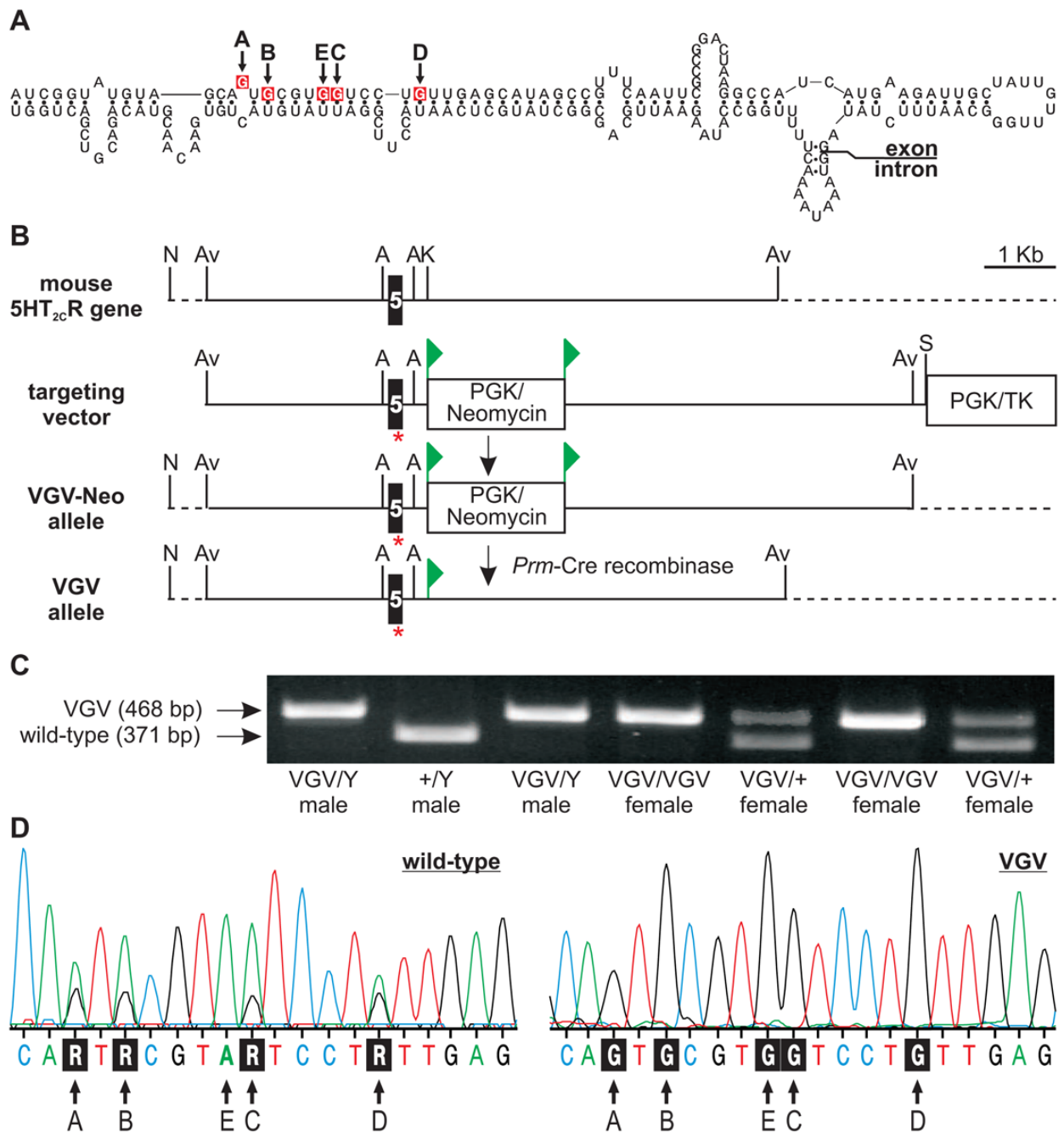
Fig. 1.
Targeting strategy and genotype analysis for 5HT$_{2C}$-VGV mice. (A) The predicted secondary structure for 5HT$_{2C}$ pre-mRNA near the distal end of exon 5 is presented. The positions of the five editing sites (A-E) are shown with site-specific adenosine to guanosine mutations indicated in red. (B) Schematic diagram and abbreviated restriction map of the mouse 5HT$_{2C}$ gene before and after targeted gene modification; the location of exon 5, the loxP sites (▶) flanking the PGK/neomycin resistance cassette, the negative selectable marker (PGK/TK) outside the region of homology, the approximate position of the introduced mutations (*) and sequences outside the region of homology (dotted line) are indicated; Av, Avr II; N, Not I; A, Acc I; K, Kpn I; S, Sfi I. (C) Genotype analysis of offspring from a

hemizygous $5HT_{2C\text{-}VGV}$ male (VGV/Y) x heterozygous mutant female (VGV/+) mating; migration positions of PCR amplicons corresponding to the wild-type (371 bp) or mutant (468 bp) $5HT_{2C}$ alleles and animal genotypes are indicated. (D) Sequence electropherogram traces of $5HT_{2C}$ receptor-derived RT-PCR products generated from wild-type and $5HT_{2C\text{-}VGV}$ hemizygous male mice. The positions of the five editing sites and the corresponding nucleotide sequences are indicated; R, purine.
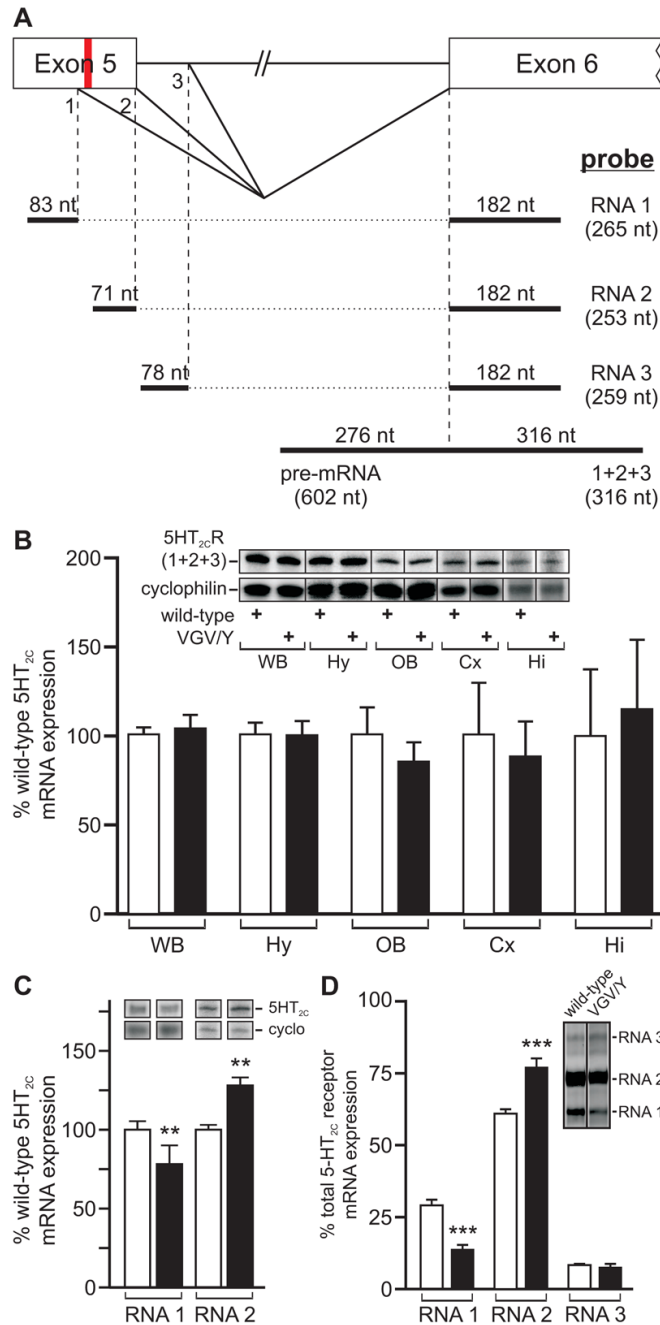
Fig. 2.
Analysis of $5HT_{2C}$ mRNA expression in wild-type and $5HT_{2C\text{-}VGV}$ mice. (A) A schematic diagram of a portion of the $5HT_{2C}$ gene is shown in which 5′-splice site selection can generate three alternatively spliced $5HT_{2C}$ transcripts (RNA 1, RNA 2 and RNA 3). The relative positions of contiguous antisense riboprobes used for ribonuclease protection analysis (bold lines) to quantify either total $5HT_{2C}$ mRNA (1+2+3) or the three individual $5HT_{2C}$ splice variants is presented with the size of the expected protected fragment (nucleotides, nt) for each mRNA isoform; the location of the 5 editing sites is indicated in red. (B) Ribonuclease protection analysis of total $5HT_{2C}$ mRNA expression (1+2+3) in whole brain and dissected brain regions for $5HT_{2C\text{-}VGV}$ (■) male mice is presented as a

percentage of mean wild-type ($\square$) expression; mean ± SEM, n ≥ 4 animals/genotype for whole brain samples and n ≥ 6 animals/genotype for dissected brain regions. *Inset,* Representative ribonuclease protection analysis of total $5HT_{2C}$ RNA expression in whole brain and dissected brain region samples from individual mice; WB, whole brain; Hy, hypothalamus; OB, olfactory bulb; Cx, frontal cortex; Hi, hippocampus. (C) Quantitative analysis of the relative expression levels for $5HT_{2C}$ alternative splicing variants (RNA 1 and RNA 2) in whole brain samples from wild-type ($\square$) and $5HT_{2C\text{-}VGV}$ ($\blacksquare$) male mice (mean ± SEM; n=7; **p ≤ 0.01). *Inset*, Representative ribonuclease protection analysis of alternatively spliced $5HT_{2C}$ RNAs in whole brain samples from individual mice. (D) Semi-quantitative analysis of $5HT_{2C}$ mRNA splicing patterns in wild-type and $5HT_{2C\text{-}VGV}$ male mice. Quantification of each $5HT_{2C}$ splice variant is presented as a percentage of total $5HT_{2C}$ mRNA expression (n=7; mean ± SEM; ***p ≤ 0.001); *inset*, Representative RT-PCR amplification using common primers in exons 5 and 6 to detect three $5HT_{2C}$ mRNA isoforms; the expected migration positions of amplicons corresponding to each $5HT_{2C}$ splice variant are indicated.
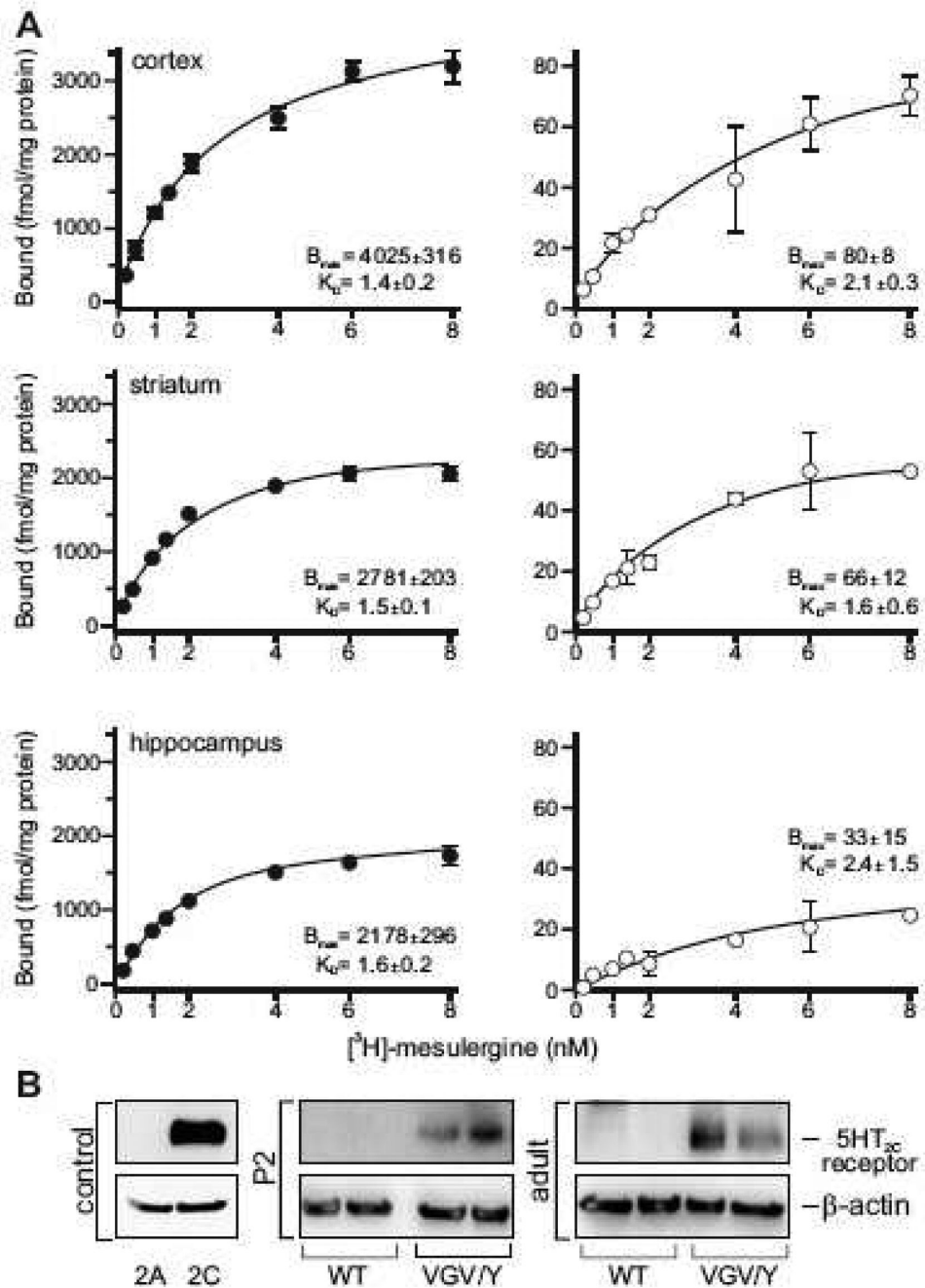
Fig. 3.
Analysis of 5HT$_{2C}$ protein expression in wild-type and 5HT$_{2C-VGV}$ mice. (A) Saturation binding isotherms demonstrating 5HT$_{2C}$ receptor-specific binding for total membranes prepared from dissected cortex, striatum and hippocampus in wild-type (□) and 5HT$_{2C-VGV}$ (●) male mice (wild-type, n=4 groups of five pooled mice per brain region; 5HT$_{2C-VGV}$, n=7 brain regions from individual animals; mean ± SEM). (B) Western blotting analysis of 5HT$_{2C}$ receptor and β-actin expression in whole brain samples isolated from individual wild-type and 5HT$_{2C-VGV}$ male mice at postnatal day 2 (P2) or in adulthood (14-16 weeks); n ≥ 8 for mice of each genotype, two representative samples are shown for each age and

genotype. Representative Western blotting controls from NIH-3T3 cells stably expressing either $5HT_{2A}$ or $5HT_{2C}$ receptors are presented.
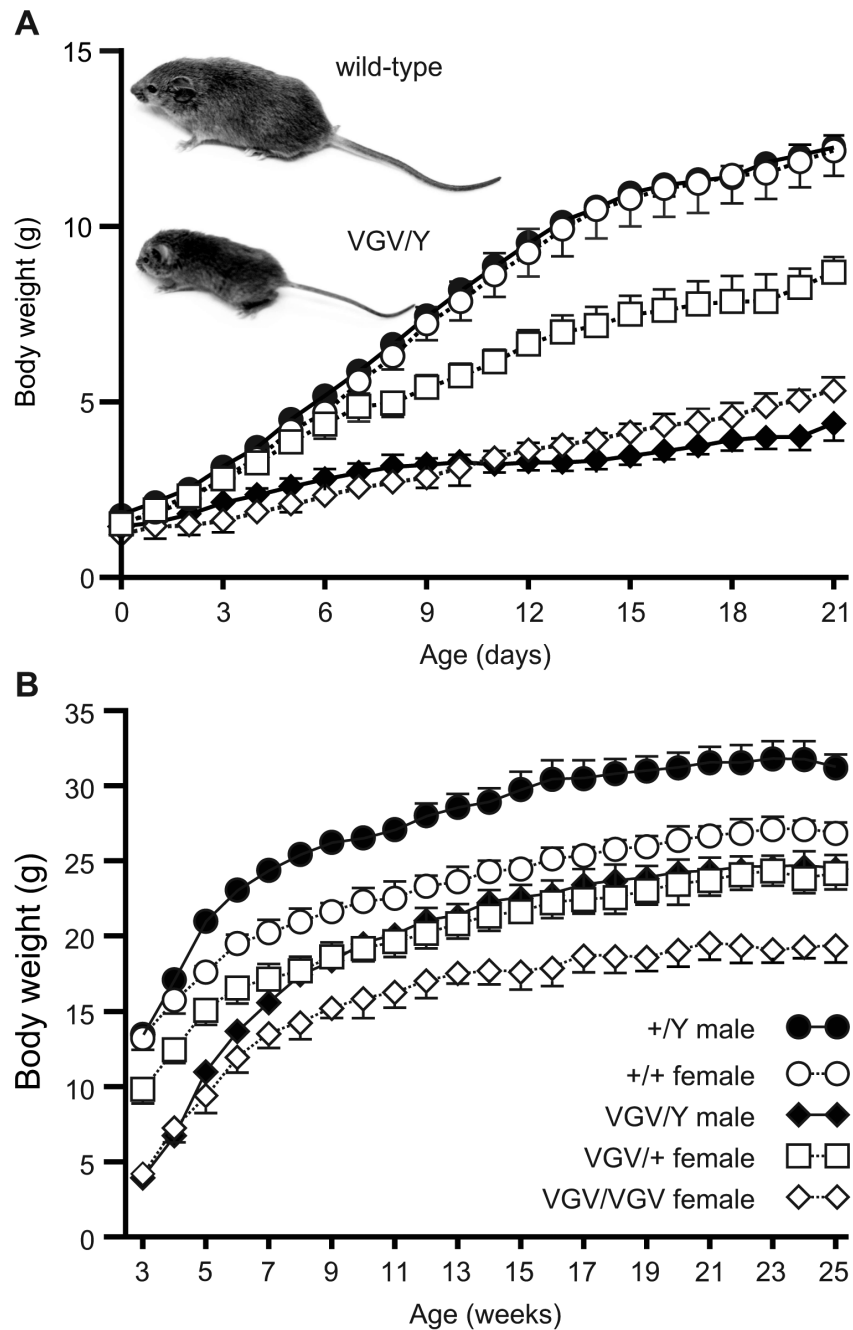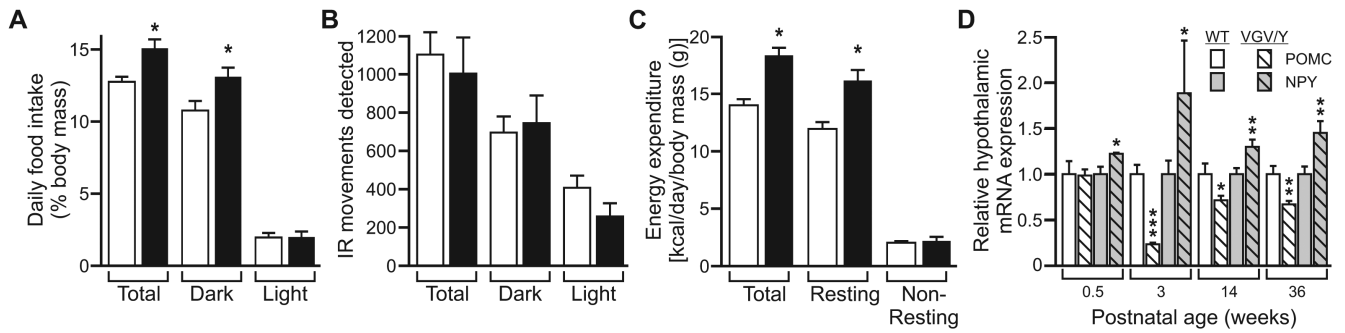
A

B

Fig. 4.
Body weight analysis of wild-type and 5HT$_{2C\text{-VGV}}$ mice. (A) Pre- and (B) post-weaning growth curves are shown for wild-type and 5HT$_{2C\text{-VGV}}$ mutant mice (mean ± SEM; n=6 for each genotype and gender; p ≤ 0.0001 using two-way ANOVA). *Inset*, A representative photograph of wild-type and mutant (VGV/Y) littermates at postnatal day 21 is presented.

Fig. 5.

Feeding behavior and metabolic analysis of wild-type and $5HT_{2C-VGV}$ mice. (A) Food intake for 15-week old male wild-type (□) and $5HT_{2C-VGV}$ (■) mice per 24-hours (hr), 12-hr light, or 12-hr dark period, corrected for total body mass (mean ± SEM, n=6, *p ≤ 0.05). (B) Average daily activity detected by infrared sensors for wild-type (□) and $5HT_{2C-VGV}$ adult (■) mice maintained in a 12hr-12hr light/dark environment (mean ± SEM; n=6). (C) Indirect calorimetric analysis of energy expenditure for wild-type (□) and $5HT_{2C-VGV}$ (■) mice per 24-hr, while resting, or while active, corrected for total body mass (mean ± SEM, n=6, *p ≤ 0.05). (D) Quantitative analysis of hypothalamic POMC and NPY mRNA expression at 0.5 weeks (n=4), 3 weeks (n=8), 14 weeks (n=12) and 36 weeks (n=11) in wild-type and $5HT_{2C-VGV}$ male mice (mean ± SEM, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001).
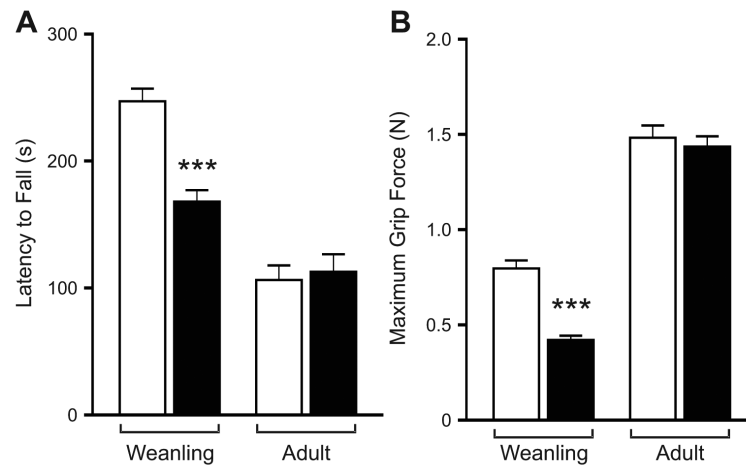
Fig. 6.
Analysis of muscle strength in wild-type and 5HT$_{2C\text{-}VGV}$ mice. (A) Motor coordination and endurance of post-weaning (P22-P28, n=10) and adult (25 weeks, n=12) wild-type (□) and 5HT$_{2C\text{-}VGV}$ mutant (■) male mice (mean ± SEM; *p ≤0.05, **p ≤ 0.01, ***p ≤ 0.001). (B) Average grip strength of recently weaned (P21-P28; n=5) or adult (n=7) wild-type (□) and mutant (■) mice (mean ± SEM; ***p ≤ 0.001).
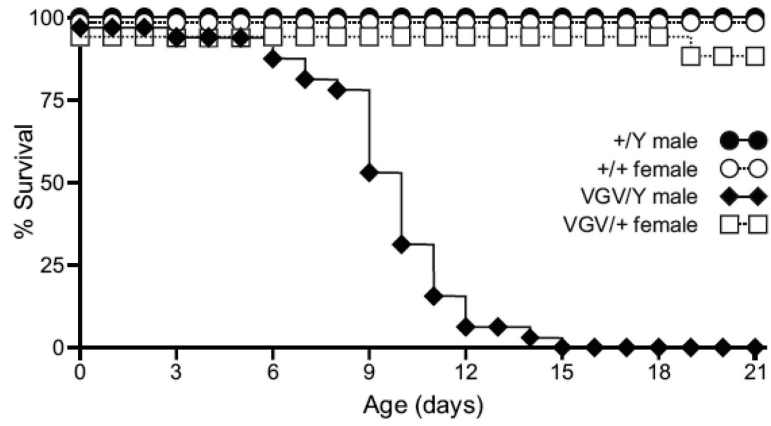
Fig. 7.
Strain-specific lethality in 5HT$_{2C\text{-}VGV}$ mice. Cumulative survival rate for offspring resulting from the mating of heterozygous mutant 129S6 females (VGV/+) to wild-type C57BL/6 males (n=28, +/Y; n=32, VGV/Y; n=33, +/+; n=17, VGV/+).

Table 1

χ^2-analysis of genotype distribution for wild-type and 5HT$_{2C-VGV}$ mutant mice.

		+/Y male × VGV/+ female			VGV/Y male × VGV/+ female		
		Genotype	Expected	Observed	Genotype	Expected	Observed
male		+/Y	24.5	21	+/Y	34.75	36
		VGV/Y	24.5	26	VGV/Y	34.75	31
female		+/+	24.5	22	VGV/+	34.75	43
		VGV/+	24.5	29	VGV/VGV	34.75	29
Total:			98	98		139	139
		p ≤ 0.65			p ≤ 0.35		

Table 2

Summary of mouse models of Prader-Willi Syndrome

Functional Chr. 7C Deletion	Mouse Model	Growth Retardation	Hypotonia	Obesity	Food Intake	C57BL/6 Lethality	Survive on Other Strains	References
Entire PWS Region	Maternal Duplication	yes	n.d.	n.d.	n.d.	100%	n.d.	[1]
Entire PWS Region	PWS-IC^del	↓~45%	n.d.	no	n.d.	100%	yes	[2,3]
Entire PWS Region	TgPWS	↓~50%	yes	n.d.	n.d.	100%	no	[4,5]
Entire PWS Region	ΔSnrpn-Ube3a	↓~55%	yes	no	n.d.	78%	n.d.	[6]
Individual Gene	ΔNecdin	no	n.d.	no	n.d.	no	yes	[7]
Individual Gene	ΔNecdin	no	yes	no	n.d.	95%	yes	[8]
Individual Gene	ΔNecdin	no	n.d.	no	n.d.	~65%	yes	[9]
Individual Gene	ΔMagel2	↓~5%	n.d.	↑~5-10%	↓~10%	prenatal*	n.d.	[10]
snoRNA	*Snord116del*	↓~40%	no	no	↑13-31%**	no	n.d.	[11]
snoRNA	ΔPWScr	↓~25%	n.d.	no	n.d.	15%	yes	[12]
N/A	5HT$_{2C\text{-}VGV}$	↓~70%	yes	no	↑14-18%	100%	yes	N/A

[1] Cattanach, 1992; [2] Yang, 1998; [3] Chamberlain, 2004; [4] Gabriel, 1999; [5] Stefan, 2005; [6] Tsai, 1999; [6] Tsai, 1999b; [7] Tsai, 1999a; [8] Gerard, 1999; [9] Muscatelli, 2000; [10] Bischof, 2007; [11] Ding, 2008; [12] Skryabin, 2007

n.d., not determined; *N/A*, not applicable

*
incomplete penetrance

**
food intake normal at 7 weeks, hyperphagia after 3 months