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Disrupted SOX10 function causes spongiform neurodegeneration in *gray tremor* mice

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Abstract

Mice homozygous for the *gray tremor* (*gt*) mutation have a pleiotropic phenotype that includes pigmentation defects, megacolon, whole body tremors, sporadic seizures, hypo- and dysmyelination of the CNS and PNS, vacuolation of the CNS, and early death. Vacuolation similar to that caused by prions was originally reported to be transmissible, but subsequent studies showed the inherited disease was not infectious. The *gt* mutation mapped to distal mouse chromosome 15, to the same region as *Sox10*, which encodes a transcription factor with essential roles in neural crest survival and differentiation. As dominant mutations in mouse or human *SOX10* cause white spotting and intestinal aganglionosis, we screened the *Sox10* coding region for mutations in *gt/gt* DNA. An adenosine to guanine transversion was identified in exon 2 that changes a highly conserved glutamic acid residue in the SOX10 DNA binding domain to glycine. This mutant allele was not seen in wildtype mice, including the related GT/Le strain, and failed to complement a *Sox10* null allele. Gene expression analysis revealed significant down-regulation of genes involved in myelin lipid biosynthesis pathways in *gt/gt* brains. Knockout mice for some of these genes develop CNS vacuolation and/or myelination defects, suggesting that their down-regulation may contribute to these phenotypes in *gt* mutants and could underlie the neurological phenotypes associated with Peripheral demyelinating neuropathy-Central dysmyelinating leukodystrophy-Waardenburg syndrome-Hirschsprung (PCWH) disease, caused by mutations in human *SOX10*.

Keywords

Sox10; spongiform neurodegeneration; vacuolation; myelination; tremor; pigmentation

Introduction

Spongiform neurodegeneration is characterized by progressive accumulation of round to oval vacuoles, varying in size up to 60 μm , throughout the central nervous system (CNS). This neuropathology is most commonly associated with prion disease, but can also be caused by infection with some viruses (including HIV), and some inherited disorders, such as Canvan disease and Leigh syndrome (Brown and Squier 1996; Budka 2003; Kumar et al. 2006; Martinez et al. 1995). Vacuolar change has also been described in more common forms of neurodegeneration, including Alzheimer's disease (Mancardi et al. 1982; Smith et al. 1987). The origin and composition of vacuoles is not well understood, nor is their role in disease pathogenesis, but the fact that they are often accompanied by gliosis, demyelination and neuron loss suggests that they are not benign.

Mice homozygous for the autosomal recessive *gray tremor* (*gt*) mutation have a pleiotropic phenotype that includes pigmentation defects (white belly, feet and tail, white forehead blaze, and genetic background-dependent dilution of pheomelanin), gastrointestinal illness, whole body tremors that start at about 8 days of age, sporadic seizures, hypo- and dysmyelination in the central and peripheral nervous systems (CNS and PNS, respectively), vacuolation of the CNS, and early death (Sidman and Cowen 1981; Sidman et al. 1985; Sweet 1981). Based on the similarities to prion disease, Sidman and co-workers tested the transmissibility of vacuolation by intracranial inoculation of wildtype mice with brain homogenates from *gt/gt* mice, with positive results (Sidman et al. 1985). Subsequent studies in the specific pathogen-free colony at McLaughlin Research Institute demonstrated that intracerebral inoculation of wildtype mice with *gt/gt* brain homogenates did not cause any signs of disease or brain pathology (Carlson et al. 1997).

Vacuoles in *gt/gt* mice were observed in CNS white and gray matter (Kinney and Sidman 1986). Electron microscopic analysis indicated that white matter vacuoles were formed by interlamellar splitting of myelin sheaths and vesicle formation in oligodendroglial inner loop cytoplasm, and that gray matter vacuoles often contained granular and membranous material (Kinney and Sidman 1986). Vacuoles were membrane-bound and ranged in size, up to 20 μm in diameter. They were reported to appear first in the white matter of the spinal cord on postnatal day seven (P7). By two weeks of age, vacuoles were observed in the gray matter of the brainstem, thalamus and spinal cord. By one month of age, vacuoles were observed throughout most of the CNS, predominantly in gray matter (Sidman et al. 1985). These observations were all made on *gt/gt* mice in a colony in which spongiform pathology was transmissible, however, raising the possibility that there may have been more than one underlying cause of CNS vacuolation and mixed pathology due to the presence of pathogenic viruses. A new analysis of the onset and distribution of CNS vacuolation in *gt* mutant mice was therefore undertaken and is reported here, along with demonstration that the *gt* phenotype is due to a loss-of-function mutation in *Sox10*. Gene expression analysis revealed reduced expression of several known SOX10 target genes, but the fact that expression of other SOX10 targets was unaffected suggests the *gt* mutation may disrupt the formation of specific transcription factor dimers. Several genes down-regulated in *gt/gt* brains are implicated in myelin lipid biosynthesis pathways, suggesting that partial SOX10 loss-of-function in *gt* mutant mice leads to CNS vacuolation through misregulation of these

genes and that a similar mechanism may underlie the neurological phenotypes associated with Peripheral demyelinating neuropathy-Central dysmyelinating leukodystrophy-Waardenburg syndrome-Hirschsprung disease (PCWH, OMIM #609136), which is caused by mutations in human *SOX10*.

Materials and Methods

Mice

Mice were maintained under standard conditions in a specific pathogen-free colony at the McLaughlin Research Institute. Before the *gt* mutation was identified, mice were genotyped by PCR using primers for the tightly linked microsatellite marker D15Mit71 (CCCAACTCATATGTATTATCCTGC and TAATGACAGTGCCAAATCTTGG). Once the mutation was identified, mice were genotyped by PCR using forward primer GGCCGAGGAACAAGACCTAT with allele-specific reverse primers CCTGGCTGACCGCCC (to detect the *gt* allele) or CCTGGCTGACCGCCT (to detect the wildtype allele). PCR was performed for 29–31 cycles using GoTaq Green 2X Master Mix (Promega) and an annealing temperature of 64 C. *Sox10^{LacZ}* mice were generated by the laboratory of Dr. Michael Wegner (Britsch et al. 2001). They were genotyped by PCR using forward primer CAGGTGGGCGTTGGGCTCTT with reverse primer CAGAGCTTGCCTAGTGTCTT (wildtype allele) or TAAAAATGCGCTCAGGTCAA (LacZ allele). All animal procedures adhered to Association for Assessment and Accreditation of Laboratory Animal Care guidelines and were approved by the Institutional Animal Care and Use Committee of the McLaughlin Research Institute.

Histology

Brains were fixed in 10% formalin for at least 1 week prior to standard processing and embedding in paraffin. Care was taken to not incubate specimens in 70% ethanol for more than 1 h and to simultaneously process mutant and control samples as prolonged exposure to 70% ethanol can cause artefactual vacuolation in rodent brain samples (Wells and Wells 1989). Sagittal or coronal sections were taken at 5 µm and stained with hematoxylin and eosin (H&E) or Luxol fast blue and eosin, or subjected to immunohistochemistry (IHC), following standard protocols. For Luxol fast blue staining, mutant and control samples were processed together to eliminate inter-experimental variability. Except for *gt/Sox10^{LacZ}*, at least 3 samples were examined for each genotype at each age, and wildtype. Rabbit anti-GFAP (ProteinTech Group 16825-1-AP), rabbit anti-SOX10 (Abcam ab25978) and mouse anti-MBP (Covance SMI99) antibodies were used for IHC. Immunolabelling was detected using horseradish peroxidase (HRP)-conjugated secondary antibodies or HRP-conjugated avidin and biotinylated secondary antibodies of the appropriate isotype (Vector Labs, CA), and peroxidase substrate diaminobenzidine (DAB, Trevigen) or NovaRed (Vector Labs). Slides were counterstained with Nuclear Fast Red (with DAB) or hematoxylin (with NovaRed) and images captured on a Zeiss AxioImagerM1 microscope using a Pixielink A623C color camera.

Mutation identification

Genomic DNA from GT/Le-*gt/gt* and GT/Le-*+/+* mice was used as template for PCR using primers to amplify all four *Sox10* exons, including exon-intron junctions (except exon 4, for which the last 58 bases were not covered, but the product included 967 bp downstream of the stop codon). Primers: CCCTTCCCTCCCTCCTTT and ACAGCCTAGGGGAGCAGAAT for exon1; AGGCCCCGAGTGGGTTTAG and CTGGGCGATCCTGTTCTG for exon 2; TCCAGGAGCCTAACTCAGA and TCTATCCAGCAGCAGCACTGG for exon 3; and ACCATCTTCTAGGGCCCACT and AAGTTAATCGGGGCTGGAGT for exon 4. PCR products were gel purified and sequenced at the Cornell University Biotechnology Resource Center. To confirm the mutation in exon 2, independent samples were generated and analyzed from 3 GT/Le-*+/+*, 3 GT/Le-*gt/+* and 3 GT/Le-*gt/gt* mice. HYIII/Le genomic DNA was obtained from the JAX DNA Resource.

X-gal staining

Sox10^{LacZ/gt} compound heterozygotes were generated from intercrossing *Sox10^{LacZ}* and *gt* heterozygotes. Compound heterozygotes were initially identified by their lack of pigmentation, which was obvious by postnatal day 4 (P4), followed by genotyping, as described above. Dorsal skin samples were dissected from compound heterozygotes and sib controls. Skins were fixed in 4% paraformaldehyde and subjected to X-gal staining to detect β -galactosidase activity following the protocol of Cepko *et al.* (<http://genepath.med.harvard.edu/~cepko/protocol/xgalplap-stain.htm>).

Gene expression studies

Total RNA was isolated from whole brain tissue from 3 GT/Le-*+/+* and 4 GT/Le-*gt/gt* mice at 3 weeks-of-age (P21–23) using Trizol reagent (Invitrogen) and analyzed on an Agilent 2100 Bioanalyzer to ensure adequate quality prior to hybridization. For each microarray experiment, 100 ng of total RNA was reverse transcribed and the resulting cDNAs were transcribed and labeled with cyanine 3-CTP. The labeled antisense cRNAs were purified and hybridized to mouse Agilent SurePrint G3 Mouse Gene Expression 8×60K microarrays that contained 39,430 Entrez Gene RNAs and 16,251 lincRNAs for 16 h at 45°C. Arrays were washed, then scanned using an Agilent scanner. The scanned images were converted and imported into the Institute for Systems Biology (ISB) System for Lab Information Management of Microarray (Marzolf and Troisch 2006). As a data pre-processing step, the sets of gene expression data were merged together and then normalized by applying the quantile normalization method using a web-based pipeline constructed at ISB.

Differentially expressed genes (DEGs) between controls and mutant samples were identified using the moderated t-test in R-package LIMMA (Smyth *et al.* 2005). P-values of genes were computed from the moderated t-statistics. DEGs were selected by considering p value (0.01 used as a cutoff) and fold-change (at least ± 1.5 -fold between *gt/gt* mutant samples and control samples). When multiple probes mapped to the same Entrez ID were identified as DEGs, only one probe with the maximum mean expression level was selected to generate a unique set 198 of DEGs. Functional analysis of DEGs was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (Huang *et al.* 2009a;

Huang et al. 2009b) to determine whether a set of genes was over-represented in a specific gene ontology (GO) term or known pathway. Gene function was also assessed using PhosphoSite (www.phosphosite.org) and AmiGO2 (amigo.geneontology.org) and the GO term finder at go.princeton.edu.

For real-time quantitative RT-PCR (qPCR), RNA was isolated from the brains of 3-week-old GT/Le-+/+ and GT/Le-*gt/gt* mice (3 of each) and treated with DNaseI (Invitrogen). Equal amounts of RNA were used as template for cDNA synthesis, followed by amplification using SYBR green Brilliant II PCR master mix (Agilent) on a BioRad Opticon II. Expression was normalized against *glucose phosphate isomerase (Gpi)* levels using the comparative C_t method to determine the relative quantification value (Livak and Schmittgen 2001). *Gpi* was included in every run, in duplicate, while other genes were analyzed in triplicate. Primer sequences are provided in Supplementary Table S1. Differences in gene expression were tested for statistical significance using a one-tailed homoscedastic *t* test to calculate a *p* value, followed by correction for multiple testing using the Benjamin and Hochberg procedure (Benjamini 1995), setting a false discovery rate (FDR) of $Q = 0.05$. Differences in expression were determined to be significant when the *t* test *p* value was less than the Benjamin and Hochberg value.

Results

The pleiotropic *gray tremor* phenotype

The earliest phenotype observed in GT/Le-*gt/gt* mice is a white forehead blaze (Fig. 1A), apparent in the skin even before hair is present. As their fur grows, additional pigmentation phenotypes become apparent in *gt/gt* animals: they lack pigment on their bellies, feet and tail tip, and pheomelanin (yellow pigment) in their coat hairs is diluted. The size and position of the forehead blaze is very consistent on the GT/Le background, but on a mixed background it is often positioned further back on the body, present as one or more stripes, or absent altogether (Supplementary Fig. S1).

The gastrointestinal illness often observed in *gt/gt* weanlings was originally described as “abdominal distension and watery fecal material with gas bubbles” (Sidman et al. 1985). Further examination revealed that *gt/gt* pups develop mild to severe megacolon (Fig. 1B). Expressivity of this phenotype was variable and penetrance incomplete, even in highly inbred animals. On the GT/Le background, the vast majority of *gt* homozygotes die by three to four weeks of age. On a mixed genetic background, rare survivors were seizure-prone and usually died suddenly, but at least one outbred female *gt/gt* mouse produced several litters and survived to one year of age.

In addition to the whole body tremor that begins around 8 days of age (Supplemental Information, Movie), widespread vacuolation is apparent in the brains of three-week-old *gt/gt* mice (Fig. 1C and Supplementary Fig. S2). As this phenotype was previously analyzed in depth in *gt/gt* animals that may also have carried a transmissible agent capable of causing spongiform change (Sidman et al. 1985), an independent analysis of the pattern and timing of vacuolation in the CNS of *gt/gt* mice from a colony free of mouse viral pathogens was performed. Vacuoles were first apparent in the pons and cerebellar white matter of *gt/gt*

mice on P9 (Fig. 2A–B and Table 1). Although the pons consistently had more severe vacuolation than the cerebellum at P9, no obvious vacuolation could be detected in either brain region on P8 (Table 1). Vacuoles appeared in the medial cerebellar nucleus, midbrain and thalamus of *gt* mutant animals between P10 and P12 (Fig. 2B–C and Table 1). Between P12 and P15, vacuoles arose in the substantia nigra, caudate putamen, corpus callosum, lateral olfactory tract and olfactory bulb, predominantly in white matter tracts (Table 1 and data not shown). By P18, mild vacuolation developed in the hippocampus and cortex (Table 1 and data not shown). In all affected brain regions, there was a rapid increase in vacuole size and number with age. Vacuolation was widespread by 3 weeks of age, with the cerebellar white matter, pons, midbrain, thalamus, caudate putamen, hippocampus, corpus callosum and olfactory bulb being most severely affected (Fig. 1C, Supplementary Fig. S2, Table 1, and data not shown). Immunohistochemistry (IHC) for glial fibrillary acidic protein (GFAP) revealed extensive gliosis in vacuolated areas of the pons and thalamus of P21 *gt/gt* mice (Fig. 1D).

The gray tremor mutation

The *gt* mutation arose spontaneously in 1977 at the Jackson Laboratory in the now extinct inbred HYIII/Le strain that carried the *hydrocephalus 3* (*hy3*) mutation (Sweet 1981). The male that produced mutant offspring was crossed with a (C3H/HeB/FeJ × C57BL/6J-*A^{w-J}*/A^{w-J}) F1 female. Intercrosses of their progeny produced mutant pups, and subsequent brother-sister matings produced the GT/Le-*gt* inbred strain. A wildtype GT/Le subline was split from the mutation-carrying strain at the 27th generation of inbreeding (F27) and maintained independently. As the *gt* mutation was previously mapped to ~19 cM from the *keratin 71* mutation *caracul* (*Krt71^{Ca}*) on mouse Chr 15 (Sidman et al. 1985), 23 microsatellite markers spanning Chr 15 were screened for polymorphism between the GT/Le-+/+ and GT/Le-*gt/gt* sublines. The two lines differed only at D15Mit68, D15Mit1 and D15Mit71 (Fig. 3A). Further screening of *gt/gt* mice and their parents indicated that both parents of all breeding pairs that produced *gt/gt* pups were heterozygous for C3H and HYIII-derived alleles at all three of these markers, and all *gt/gt* mice were homozygous for the HYIII-derived allele at D15Mit71 (Chr 15: 79,590,436–79,590,561). In a subsequent analysis, 40 of 347 offspring from parents both heterozygous at D15Mit71 had the *gt* phenotype; departure from Mendelian expectations is consistent with reduced viability of *gt* homozygotes. All 40 *gt/gt* mice were homozygous for the HYIII-derived allele at D15Mit71 and all phenotypically normal mice were heterozygous or homozygous for the C3H-derived allele. The 8 mice with recombination breakpoints between D15Mit68 and D15Mit71 were typed for polymorphic intervening markers, which placed *gt* distal to D15Mit146 and D15Mit1 (Fig. 3B). D15Mit146 maps to Chr 15: 77,257,787–77,257,911. D15Mit1 is not included in the current mouse genome database (GRCm38/mm10) but BLAST search mapped it to Chr 15: 78,716,894–78,717,095.

The *gt* candidate interval contained 63 protein-coding genes, 15 RNA-coding genes and 5 processed transcripts. Given the white spotting and megacolon phenotypes of *gt/gt* mice, an obvious candidate gene was *SRY* (*sex determining region Y*)-*box 10* (*Sox10*), which maps within 450 kb of D15Mit79, at Chr 15: 79,154,913–79,164,490 Mb. Dominant mutations in human *SOX10* (usually causing premature termination) cause Waardenburg-Shah syndrome,

also known as Waardenburg syndrome type 4 (WS4C) (Pingault et al. 1998). WS4C is characterized by deafness, pigmentary abnormalities, and Hirschsprung disease (aganglionic megacolon). Similarly, a dominant premature termination mutation in mouse *Sox10* (*Sox10^{Dom}*) causes white spotting and megacolon (Herbarth et al. 1998; Southard-Smith et al. 1998). The *Sox10* coding region was amplified from GT/Le-*gt/gt* genomic DNA and the PCR products sequenced. In samples from *gt/gt* mice, a single base-pair change (A to G) was identified at nucleotide 299 of exon 2, corresponding to position 221 of the coding region (Fig. 3C). Sequencing of co-isogenic wildtype and heterozygous GT/Le mice and allele-specific PCR of HYIII/Le strain DNA confirmed that the *gt* mutation arose on a strain background that carries the wildtype sequence (adenine) and that heterozygotes carry both alleles (Fig. 3C–D). Ongoing genotyping of colony animals using allele-specific primers has shown perfect cosegregation of the G allele with the *gt* phenotype. This transversion alters a glutamic acid at amino acid position 74, within in the SoxE dimerization domain, to a glycine residue (Fig. 3E). This residue is highly conserved, being a glutamic or aspartic acid in all sequenced vertebrate SOX10 orthologs and all mouse SoxE paralogs (Fig. 3F). Real time qRT-PCR of P21 brain RNA showed no significant difference in *Sox10* expression between GT/Le-+/+ and GT/Le-*gt/gt* mice (relative expression 1.0 (0.7–1.5) and 1.2 (1.0 – 1.5), respectively, $p = 0.24$). IHC indicated comparable numbers of SOX10-positive cells with a similar pattern of staining in the thalamus of wildtype and *gt/gt* brains at P15 and P21 (Supplementary Fig. S3).

The *gt* mutation causes SOX10 loss-of-function

Mice heterozygous for the *gt* mutation were previously reported to develop mild to moderate vacuolation in CNS gray matter starting at approximately two months of age (Sidman et al. 1985). In our colony, mild vacuolation was observed predominantly in cerebellar, hindbrain and thalamic white matter of *gt/+* mice after seven months of age (Fig. 4A). A similar pattern of mild vacuolation was observed in the brains of mice heterozygous for the *Sox10^{tm1Weg}* (*Sox10^{lacZ}*) null allele, also starting between seven and eight months of age (Fig. 4A). Vacuoles were not observed in the brains of wildtype mice at any age examined. These results are consistent with the *gt* mutation causing loss of SOX10 function.

To further investigate the effect of the *gt* mutation on SOX10 function, mice carrying the *gt* mutation were mated to animals heterozygous for the *Sox10^{lacZ}* allele to generate compound heterozygotes. Most *Sox10^{lacZ}* homozygotes die late in gestation, and those that survive to birth die perinatally due to a failure to breathe (Britsch et al. 2001). *Sox10^{lacZ}/gt* compound heterozygotes survived to birth, but most died within four to five days. They had pigmented eyes but white fur (Fig. 4B–D), and all developed severe and invariably fatal megacolon (Fig. 4D). As the *Sox10^{LacZ}* mutation is a knock-in of *LacZ* into the *Sox10* locus, X-gal staining was performed on the skin of P4 *Sox10^{LacZ}/+* heterozygotes and *Sox10^{lacZ}/gt* compound heterozygotes to determine whether the lack of hair pigment in the latter reflected a lack of melanocytes, or whether melanocytes were present but unable to make pigment. Skin from mice of both genotypes showed staining of peripheral nerves, but the dots of staining that represent melanocytes in the *Sox10^{LacZ}/+* skin were absent from *Sox10^{lacZ}/gt* skin (Fig. 4B). Histologic analysis of the brain of a rare *Sox10^{lacZ}/gt* mouse that survived to P8 revealed no obvious spongiform degeneration, while the brain of the only compound

heterozygote that survived beyond that age, to P10, had mild vacuolation in the pons and cerebellar white matter, similar to that observed in P10 *gt/gt* mice (Fig. 4E–F). These data are consistent with *gt* being a strongly hypomorphic *Sox10* allele and suggest that reduction of SOX10 function below that maintained in *gt/gt* mice does not cause an earlier onset of vacuolation.

Myelin lipid metabolism genes are down-regulated in *gt/gt* brains

SOX10 plays a key role in the survival of oligodendrocyte precursor cells and their terminal differentiation into mature, myelinating oligodendrocytes (Finzsch et al. 2008; Pozniak et al. 2010; Stolt et al. 2002; Takada et al. 2010). To identify genes and pathways affected by the *gt* mutation, unbiased, global gene expression analysis was performed by hybridizing RNA isolated from the brains of 3-week-old GT/Le-+/+ and GT/Le-*gt/gt* mice to mouse Agilent arrays that contained 39,430 Entrez Gene RNAs and 16,251 lincRNAs. A total of 198 transcripts showed at least a 1.5-fold difference in expression between *gt/gt* and wildtype brains, significant at $p < 0.01$ (Supplementary Table S2 and Fig. S4A). Of these, 81 were up-regulated and 117 were down-regulated. Pathway analysis using DAVID did not reveal significant enrichment for any biological process gene ontology (GO) terms for the genes up-regulated in *gt/gt* brains, but other analyses indicated enrichment for terms related to cell motility, response to stimulus, cellular protein metabolic process and signal transduction. A number of up-regulated genes were also implicated in stress response and immune system processes, which suggests they may be secondary changes in response to CNS damage and/or dysfunction.

Of greater interest were the down-regulated genes, given that our data indicate the *gt* mutation leads to reduced SOX10 function and would therefore be expected to result in lower expression of SOX10 targets. Lipid biosynthesis and metabolism, cellular ion homeostasis, regulation of membrane potential, organic acid biosynthesis, and ensheathment of neurons were the most significantly enriched biological process GO terms for genes down-regulated in *gt/gt* brains (Table 2 and Supplementary Table S2). This gene set included some known SOX10 targets: *myelin basic protein (Mbp)*, *gap junction protein, beta 1 (Gjb1)*, encodes connexin 32) and *gap junction protein, gamma 2 (Gjc2)*, encodes connexin 47) (Bondurand et al. 2001; Schlierf et al. 2006). Several other significantly down-regulated genes have known roles in oligodendrocyte development, myelination and/or lipid metabolism (Supplementary Fig. S4B), including *fatty acid 2-hydroxylase (Fa2h)*, *kallikrein-related peptidase 6 (Klk6)*, *peptidyl arginine deiminase, type 2 (Padi2)*, *transferrin (Trf)*, and *UDP galactosyltransferase 8A (Ugt8a)*. These 8 genes, plus 4 other down-regulated genes and the 4 most highly up-regulated ones were selected for validation in brain RNA isolated from an independent set of three-week-old wildtype and *gt/gt* mice animals. Real-time qRT-PCR confirmed that *Cysteine-rich secretory protein 3 (Crisp3)*, *Polycystic kidney disease 2-like 1 (Pkd2l1)*, *Cysteine-rich secretory protein 1 (Crisp1)* and *Tumor necrosis factor alpha induced protein 6 (Tnfaip6)* were significantly up-regulated and *Gjb1*, *Klk6*, *ATPase, class V, type 10B (Atp10b)*, *Fa2h*, *Ninjurin (Ninj2)*, *Trf*, and *Ugt8a* were significantly down-regulated in the brains of *gt/gt* mice (Table 3). Expression of *Mbp*, *Gjc2* and *Gjc3* was not significantly different between wildtype and *gt/gt* brains by qRT-PCR. Although *Padi2* expression differed significantly, it was down-regulated in the microarray

data but up-regulated in the qRT-PCR analysis. A search of the Prion Disease Database indicates that very few of these genes nor *Sox10* itself were significantly perturbed in prion disease (Gehlenborg et al. 2009; Hwang et al. 2009). SOX10 loss-of-function and prions both appear to disrupt cholesterol metabolism and sterol biosynthesis processes, however (Table 2 and Cui et al. 2014; Kumar et al. 2008), suggesting there may be some shared pathways that contribute to CNS vacuolation.

To confirm that expression of other known SOX10 target genes was not altered in *gt/gt* brains, real-time qRT-PCR was performed for *proteolipid protein (myelin) 1 (Plp1)*, *myelin associated glycoprotein (Mag)* and *2',3'-Cyclic-nucleotide 3'-phosphodiesterase (Cnp)*. All three showed suggestive, but not significant, reductions in expression in *gt/gt* brains (Table 3). The failure to observe significant changes in the expression of several known SOX10 targets may reflect functional redundancy between SOX10 and related SOXE proteins, or retention of sufficient SOX10 function in *gt/gt* brains to meet the threshold required to activate transcription of many of its targets.

CNS myelination is severely disrupted in *gt/gt* mice

Several of the genes down-regulated in *gt/gt* brains are involved in processes that affect myelination, especially lipid biosynthesis and metabolism. To test whether myelin production is affected by the *gt* mutation, wildtype and *gt/gt* brain sections were stained with Luxol fast blue, which binds myelin lipoproteins. While robust staining was observed in wildtype P15 and P21 brains, staining was almost undetectable in *gt/gt* brains (Fig. 5A–D). This indicates a substantial defect in the production of normal, mature myelin in *gt/gt* brains despite normal expression of genes encoding myelin components such as *Mbp*, *Mag*, *Cnp* and *Plp1*. IHC for MBP confirmed similar expression levels in the CNS of wildtype and *gt/gt* mice on P15 (Fig. 5E–J). Despite no difference in *Mbp* RNA levels, IHC indicated consistently reduced levels of MBP protein in *gt/gt* brains by P21 (Fig. 5K–P), suggesting there may be an age-dependent post-transcriptional effect of the *gt* mutation on MBP levels. These data demonstrate that *gt/gt* mutant mice produce at least some myelin components, but their ability to generate mature myelin is impaired.

Discussion

Our data indicate that *gt* is a loss-of-function mutation in *Sox10*. The glutamic acid at position 74 of the SOX10 protein that is altered to glycine by the *gt* mutation is a negatively charged, strongly polar glutamic or aspartic acid in vertebrate species. Glycine is considered weakly or not polar, is uncharged, and contains only hydrogen as its side chain, giving it greater conformational flexibility than other amino acids. The effect of this substitution on SOX10 function is difficult to predict, but the adjacent cysteine, isoleucine and arginine, at positions 71–73, play a critical role in SOX10 dimerization: replacing them with alanines reduced the cooperative binding of SOX10 to two adjacent target sites in the *myelin protein zero (Mpz)* promoter (Schlierf et al. 2002). Mice heterozygous for a knock-in of this mutation (*Sox10^{CIR71-73AAA}*) were grossly normal, except for a white belly spot (Schreiner et al. 2007), while homozygotes developed severe megacolon and died within 3 days of birth. Homozygotes also had a significant reduction in enteric neurons and glia, a virtual absence

of melanocytes, and persistence of PNS glia at an immature stage. No significant defects in oligodendrocyte specification or differentiation or ability to form myelin were observed in the spinal cord of *Sox10*^{CIR71-73AAA} homozygotes by embryonic day 18.5, but they were not analyzed at later ages and died before the age at which CNS vacuolation is observed in *gt/gt* mice.

Unlike most *Sox10* mutations, the *gt* allele only causes pigmentation defects in homozygotes, while mild megacolon and CNS vacuolation are observed in some *gt* heterozygotes. Waardenburg-Hirschsprung syndrome is thought to be a result of loss of SOX10 function (with causative mutations causing nonsense-mediated decay of the *SOX10* transcript), while dominant missense mutations in human *SOX10* exons 3 and 4 cause a neurological variant of Waardenburg-Shah syndrome, PCWH. While PCWH-associated mutations are generally thought to reflect gain-of SOX10 function, the identification of at least two patients carrying deletions that encompass *SOX10* suggests that reduced *SOX10* expression can disrupt myelination (Bondurand et al. 2007). Our analysis of *gt/gt* mice also suggests that reduced SOX10 function can cause neurological phenotypes. Our study reveals a severe myelination defect in the CNS of *gt/gt* mice, consistent with SOX10 being required for normal oligodendroglial differentiation and the production and/or maintenance of myelin.

Normal expression levels of oligodendrocyte-specific genes such as *Sox10*, *Olig1*, *Olig2*, *Mbp*, *Cnp* and *Plp1* in the brains of *gt/gt* mice suggests that sufficient SOX10 function is maintained to form oligodendrocytes capable of generating at least some myelin components, but not mature myelin. Reduced expression of *Gjb1*, a known SOX10 target (Bondurand et al. 2001), may provide insight into the mechanism by which the *gt* mutation causes CNS vacuolation and hypomyelination. *Gjb1* encodes connexin 32, and mice lacking both connexin 32 and connexin 47 mice show vacuolation within myelinated regions of the CNS, myelin abnormalities, an action tremor and tonic seizures (Menichella et al. 2003; Odermatt et al. 2003). While 4–5-fold reduction in *Gjb1* expression is unlikely to be sufficient on its own to cause these phenotypes, our microarray data suggest that the *gt* mutation may also cause a reduction in *Gjc2* (which encodes connexin 47), although the difference was not significant when assessed by qRT-PCR.

Mice homozygous for the *gt* mutation also have reduced expression of other genes that affect the production or organization of myelin components, which could act epistatically with reduced *Gjb1* levels to cause CNS vacuolation, hypomyelination and tremor. For example, PADI2 catalyzes the post-translational deimination of arginine residues into citrullines on target proteins. One of its targets is MBP, deimination of which results in a significant alteration of its topology and lipid-organizing and self-interaction properties (Harauz and Musse 2007; Ishiyama et al. 2001). Altered *Padi2* expression might therefore explain why *gt/gt* mice produce substantial amounts of MBP but little mature myelin. Genes implicated in myelin lipid biosynthesis and metabolism were also among those consistently down-regulated in the brains of *gt/gt* mice. Among these, two encode proteins involved in the biosynthesis of major myelin lipids: *Ugt8a* and *Fa2h*. UGT8A catalyzes the transfer of galactose to ceramide, a key step in the biosynthesis of galactocerebrosides, which are among the most abundant myelin lipids. *Ugt8a* null mice produce abnormal myelin and, like

gt/gt mice, exhibit a tremor and CNS vacuolation (Coetzee et al. 1996; Marcus et al. 2000). FA2H is responsible for formation of alpha-hydroxylated ceramide (the most abundant lipid in the myelin sheath) in oligodendrocytes (Alderson et al. 2004; Eckhardt et al. 2005). In humans, homozygosity for *FA2H* loss-of-function mutations causes leukodystrophy with spastic paraparesis and brain iron accumulation (Edvardson et al. 2008). Mice lacking FA2H initially form structurally normal myelin, despite lacking 2-hydroxylated sphingolipids, but develop late-onset axon and myelin degeneration, indicating a role for FA2H in myelin maintenance (Potter et al. 2011; Zoller et al. 2008).

While oligodendrocytes capable of producing at least some myelin proteins are present in *gt/gt* mice, it is possible that the mutation disrupts their differentiation or maturation. Two genes consistently down-regulated in the brains of *gt/gt* mice have been implicated in oligodendrocyte development. The most highly down-regulated gene was *Klk6*, which encodes one of two serine proteases constitutively and abundantly expressed in oligodendrocytes. Knock-down of *Klk6* expression in cultured oligodendrocytes led to reduced *Mbp* and *Plp1* expression (Bando et al. 2006), suggesting a role for KLK6 in myelination. *Klk6* null mice had reduced MBP levels and fewer oligodendrocytes in their spinal cord during development, but not in adult mice (Murakami et al. 2013). *Trf* was also down-regulated in *gt/gt* brains. It encodes an iron-binding and transport protein expressed in oligodendrocytes in the CNS. Iron is required as a cofactor in the synthesis of myelin lipids, and developmental iron deficiency in rats resulted in reduced MBP, PLP, lipids and cholesterol (Ortiz et al. 2004; Yu et al. 1986). Intracranial injection of iron-deficient rats with TRF stimulated myelination, indicating TRF levels and overall iron homeostasis may be more critical for normal myelin production than iron levels (Badaracco et al. 2008). While the CNS effects of reduced *Trf* expression are not known, transgenic mice over-expressing human *TRF* showed accelerated oligodendrocyte differentiation associated with increased levels of SOX10 and myelin components (Espinosa de los Monteros et al. 1994; Saleh et al. 2003).

Taken together, our results suggest that reduced expression of a number of genes may act together to disrupt oligodendrocyte differentiation and/or myelin production in the CNS of *gt/gt* mice. Vacuoles first appear in white matter tracts of the cerebellum and hindbrain of *gt/gt* mice. Although they later spread to some areas of gray matter, they continue to be most severe in the white matter and associated with MBP immunoreactivity. The onset of vacuolation appears to correlate with the initiation of myelination in the CNS. Given that neuronal activity promotes myelination, vacuolation in *gt/gt* mice may be activity-dependent, similar to what was reported for connexin 32/47 double mutant mice (Menichella et al. 2006). Identifying the mechanism by which the *gt* mutation causes these phenotypes may provide insight into the basis –and treatment– of the neurological phenotypes associated with PCWH in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Phenotypes observed in 3-week-old GT/Le-*gt/gt* mice. (A–B) Pigmentation phenotypes of GT/Le-*gt/gt* mice include dilution of pheomelanin (yellow pigment) in coat hairs, giving them a grayish appearance, a white forehead blaze, a white belly and white feet. (C) Intestines of two *gt/gt* mice, showing one with megacolon and an age-matched sibling that is unaffected. (D) H&E-stained paraffin sections of the brains of GT/Le-+/+ (wildtype) and GT/Le-*gt/gt* animals showing typical pattern of vacuolation in *gt/gt* cerebellum, pons, thalamus and hippocampus. ML: molecular layer, GL: granule layer, WM: white matter. All panels at same magnification, scale bar: 5 μ m. (E) CNS vacuolation in *gt/gt* brains is associated with gliosis, as revealed by IHC for GFAP. All panels at same magnification, scale bar: 10 μ m.

Fig. 2.

Time-course of CNS vacuolation in *gt/gt* mice. (A–C) H&E-stained paraffin sections of the pons (A), cerebellum (B) and thalamus (C) of *gt/gt* and wildtype mice at postnatal day 9 (P9), P10, P12 and P15. Vacuoles first appear in the pons and cerebellar white matter on P9, and in the thalamus between P10 and P12. In all brain regions, vacuoles become more numerous and larger with age. All panels at same magnification, scale bars: 10 μ m.

Fig. 3.

Mapping and identification of the *gt* mutation. (A) Linkage map of Chr 15 showing microsatellite markers assessed and the region (in red) inherited from HYIII in GT/Le-*gt/gt* mice and from C3H in GT/Le-*+/+* (wildtype) mice (right). (B) Fine mapping of the region shown in red in panel A in mice segregating for the *gt* allele. HYIII and C3H alleles denoted by H and C, respectively. Markers for which genotype is consistent with known or presumed *gt* genotype are boxed in red, while those showing recombinant genotypes are boxed in blue. Marker location on the physical map (GRCm68) are given below each marker name. (C) Sequencing chromatograms of *Sox10* exon 2 PCR products amplified from genomic DNA of wildtype, *gt/+* and *gt/gt* GT/Le mice, showing heterozygosity for the A to G transversion in *gt/+* mice and homozygosity for the G allele in *gt/gt* mice. (D) PCR products obtained using allele-specific primers for the wildtype (*wt*) and *gt* alleles, showing presence of the wildtype product in wildtype and heterozygous GT/Le mice and the HYIII/Le strain and the *gt* allele only in *gt* heterozygotes and homozygotes. (E) Schematic of SOX10 protein showing conserved protein motifs: DNA-dependent dimerization domain (DIM), high mobility group domain (HMG), K2 region (conserved between SOXE proteins but of unknown function), and transactivation domain (TAD). The *gt* mutation changes a glutamic acid in the dimerization domain to a glycine, shown in red. (F) Partial amino acid sequence of SOX10 DIM showing that the glutamate altered by the *gt* mutation (in red) is highly conserved between SOX10 proteins across vertebrate species. Mouse SOXE paralogs, SOX8 and SOX9, also carry either a glutamic or aspartic acid at this position.

Fig. 4.

The *gt* mutation causes SOX10 loss-of-function. (A) H&E-stained paraffin sections of 12-month-old *gt*/+ (*gt* het) and *Sox10^{LacZ}/+* (*Sox10^{LacZ} het*) mice, showing mild CNS vacuolation in the pons, cerebellum and thalamus, and 12-month-old wildtype mice, showing no vacuolation. Scale bars: 10 μ m. (B) A P4 *gt/Sox10^{LacZ}* pup shown with its *Sox10^{LacZ}/+*, *gt*/+ and wildtype siblings. (C) Lack of pigmentation in the compound heterozygote reflects a lack of skin melanocytes, as shown by X-galactosidase staining (C). (D) *gt/Sox10^{LacZ}* compound heterozygotes exhibit a distended belly, indicative of megacolon. (E–F) H&E-stained paraffin sections of the pons and cerebellum of P10 *gt/gt* and *Sox10^{LacZ}/gt* mice, with both showing a similar distribution and severity of vacuoles. For wildtype P10 comparison, see Fig. 1A–B. Scale bars: 10 μ m.

Fig. 5. Hypomyelination in *gt/gt* brains. (A–D) Luxol fast blue staining on P15 (A,B) and P21 (C,D) wildtype (A,C) and *gt/gt* (B,D) cerebellum sections shows greatly reduced staining, indicating reduced myelin lipoproteins, in *gt/gt* mice. Scale bars: 10 μ m. (E–J) IHC for MBP (blue DAB staining) on P15 brain sections shows similar levels of staining in wildtype (E–G) and *gt/gt* (H–J) hippocampus (E, H), thalamus (E, F, H, I) and cerebellum (G, J). cc: corpus callosum. Scale bars: E, H: 20 μ m; F, I: 10 μ m; G, J: 5 μ m. (K–P) IHC on P21 wildtype (K–M) and *gt/gt* (N–P) brain sections shows reduced levels of MBP (NovaRed staining) in the cortex and corpus callosum (K, N), thalamic white matter (L, O) and cerebellum (M, P). cc: corpus callosum, wm: cerebellar white matter. Scale bars: 10 μ m.

Table 1

Developmental sequence and severity of CNS vacuolation[†] in *gt/gt* mice.

Brain region	P8	P9	P10	P12	P15	P18	P21
Medulla	ns	NA	mod	sev	sev	sev	sev
Hindbrain (pons)	ns	mod	mod	sev	sev	sev	sev
Cerebellum:							
White Matter	ns	mod	mod	sev	sev	sev	sev
Granule Layer	ns	ns	ns	ns	rare	rare	rare
Molecular Layer	ns	ns	ns	ns	ns	ns	ns
Midbrain	ns	ns	ns	rare	mod	sev	sev
Substantia Nigra	ns	ns	ns	ns	mod	sev	sev
Thalamus	ns	ns	ns	mild	mod	sev	sev
Hippocampus	ns	ns	ns	ns	ns	ns	mild
Amygdala	ns	ns	ns	ns	ns	ns	ns
Cortex	ns	ns	ns	ns	ns	mild	mod
Corpus Callosum	ns	ns	ns	ns	mod	sev	sev
Caudate Putamen	ns	ns	ns	ns	rare	rare	mild
Ventral Striatum	ns	ns	ns	ns	ns	mild	mod
Piriform Cortex	ns	ns	ns	ns	ns	ns	ns
Lateral Olfactory Tract	ns	ns	ns	sev*	sev*	sev*	sev*

[†] Scoring was based on average number of vacuoles in field of view at 20X magnification. ns = no significant lesions (0–2 vacuoles per field of view); rare = few, scattered vacuoles (3–5 vacuoles); mild = mild vacuolation (6–10 scattered vacuoles); mod = moderate vacuolation (11–25 evenly scattered vacuoles); sev = severe vacuolation (> 25 vacuoles with confluence). NA: not scored.

Table 2Gene ontology classifications of genes down-regulated in *gt/gt* brains.

Biological process gene ontology term (BP_FAT) [†]	Fold enrichment	Genes	Benjamini p value
GO:0008610~lipid biosynthetic process	7.9	SCD1, ELOVL1, CYP39A1, UGT8A, FA2H, HMGCS1, MBOAT1, ELOVL7, IDI1, SC4MOL, DHCR24	0.0004
GO:0016125~sterol metabolic process	16.0	CYP39A1, LDLR, HMGCS1, IDI1, SC4MOL, DHCR24	0.0076
GO:0042592~homeostatic process	4.2	LDLR, UGT8A, CLDN11, KCNA5, GJC3, TRF, MBP, HBA-A1, HBA-A2, ALAS2, XIRP1, AHSP, COL11A2	0.0141
GO:0006873~cellular ion homeostasis	6.3	ALAS2, XIRP1, UGT8A, KCNA5, CLDN11, GJC3, TRF, MBP	0.0277
Shared terms:			
GO:0055082~cellular chemical homeostasis	6.1		0.0218
GO:0050801~ion homeostasis	5.6		0.0203
GO:0019725~cellular homeostasis	4.8		0.0351
GO:0042391~regulation of membrane potential	10.4	XIRP1, UGT8A, KCNA5, CLDN11, GJC3, MBP	0.0238
GO:0048878~chemical homeostasis	5.1	ALAS2, LDLR, XIRP1, UGT8A, KCNA5, CLDN11, GJC3, TRF, MBP	0.0221
GO:0008203~cholesterol metabolic process	14.7	CYP39A1, LDLR, HMGCS1, IDI1, DHCR24	0.0200
GO:0006694~steroid biosynthetic process	14.5	CYP39A1, HMGCS1, IDI1, SC4MOL, DHCR24	0.0188
GO:0016126~sterol biosynthetic process	27.5	HMGCS1, IDI1, SC4MOL, DHCR24	0.0180
GO:0016053~organic acid biosynthetic process	8.8	SCD1, ELOVL1, CYP39A1, FA2H, ELOVL7, SC4MOL	0.0217
Shared term:			
GO:0046394~carboxylic acid biosynthetic process	8.8		0.0217
GO:0006633~fatty acid biosynthetic process	12.7	SCD1, ELOVL1, FA2H, ELOVL7, SC4MOL	0.0215
GO:0007272~ensheathment of neurons	21.1	UGT8A, CLDN11, GJC3, MBP	0.0279
Shared terms:			
GO:0008366~axon ensheathment	21.1		0.0279
GO:0019228~regulation of action potential in neuron	18.3		0.0348
GO:0001508~regulation of action potential	15.3		0.0552
GO:0008202~steroid metabolic process	7.7	CYP39A1, LDLR, HMGCS1, IDI1, SC4MOL, DHCR24	0.0315

[†]GO terms grouped when related terms were represented by the same set of genes; terms with lower fold-enrichment are given in parenthesis below the GO term with the highest fold-enrichment. Only terms with Benjamini-corrected $p < 0.05$ are shown.

Table 3

Expression of SOX10 targets and DEGs in P21 wildtype and *gt/gt* brains.

Gene	Relative expression [†] (range)		p value (t test)	BH [§]
	Wildtype	gt/gt		
DEGs				
<i>Klk6</i>	1.0 (0.82 – 1.22)	0.09 (0.06 – 0.13)	0.0003	0.003 [*]
<i>Pkd2l1</i>	1.0 (0.50 – 2.00)	43.1 (39.3 – 47.3)	0.00037	0.005 [*]
<i>Atp10b</i>	1.0 (0.74 – 1.35)	0.04 (0.02 – 0.11)	0.001	0.008 [*]
<i>Fa2h</i>	1.0 (0.88 – 1.14)	0.45 (0.37 – 0.55)	0.002	0.011 [*]
<i>Trifaip6</i>	1.0 (0.52 – 1.92)	11.0 (7.7 – 15.9)	0.0025	0.013 [*]
<i>Crisp3</i>	1.0 (0.44 – 2.28)	124 (112.9 – 136.0)	0.003	0.016 [*]
<i>Gjb1</i>	1.0 (0.72 – 1.39)	0.20 (0.14 – 0.30)	0.0077	0.018 [*]
<i>Crisp1</i>	1.0 (0.52 – 1.90)	87.7 (13.3 – 579)	0.0089	0.021 [*]
<i>Ninj2</i>	1.0 (0.53 – 1.91)	0.08 (0.03 – 0.19)	0.011	0.024 [*]
<i>Trf</i>	1.0 (0.71 – 1.40)	0.44 (0.35 – 0.54)	0.011	0.026 [*]
<i>Padl2</i>	1.0 (0.70 – 1.43)	3.91 (3.19 – 4.78)	0.012	0.029 [*]
<i>Ugt8a</i>	1.0 (0.79 – 1.26)	0.57 (0.45 – 0.73)	0.023	0.032 [*]
<i>Gjc2</i>	1.0 (0.41 – 2.44)	0.53 (0.41 – 0.67)	0.15	0.042
<i>Alas2</i>	1.0 (0.54 – 1.85)	0.77 (0.45 – 1.33)	0.31	0.045
<i>Mbp</i>	1.0 (0.59 – 1.71)	0.81 (0.51 – 1.29)	0.31	0.047
<i>Gjc3</i>	1.0 (0.55 – 1.81)	1.09 (1.00 – 1.19)	0.43	0.050
SOX10 targets				
<i>Mag</i>	1.0 (0.79 – 1.27)	0.62(0.41 – 0.94)	0.08	0.043
<i>Cnp</i>	1.0 (0.80 – 1.25)	0.57 (0.36 – 0.91)	0.07	0.043
<i>Pip1</i>	1.0 (0.54 – 1.86)	0.45 (0.39 – 0.51)	0.05	0.037

[†] CT method, normalized against *Gpi*, with wildtype set to 1.0.

[§] Benjamin & Hochberg value, using false discovery rate of 0.05.

* *gt/gt* expression significantly different from wildtype ($p < BH$).