Dominant mutations of the P/Q-type Ca\(^{2+}\) channel (CACNA1A) underlie several human neurological disorders, including episodic ataxia type 2, familial hemiplegic migraine 1 (FHM1) and spinocerebellar ataxia 6, but have not been found previously in the mouse. Here we report the first dominant ataxic mouse model of Cacna1a. This Wobbly mutant allele of Cacna1a was identified in an ethyl-nitrosourea (ENU) mutagenesis dominant behavioral screen. Heterozygous exhibit ataxia from 3 weeks of age and have a normal life span. Homozygous mice show a righting reflex defect from post-natal day 8 and later develop severe ataxia and die prematurely. Both heterozygote and homozygote exhibit cerebellar atrophy with focal reduction of the molecular layer. No obvious loss of Purkinje cells or decrease in size of the granule cell layer was observed. Real-time polymerase chain reaction revealed altered expression levels of Cacna1g, Calb2 and Th in Wobbly cerebella, but Cacna1a messenger RNA and protein levels were unchanged. Positional cloning revealed that Wobbly mice have a missense mutation leading to an arginine to leucine (R1255L) substitution, resulting in neutralization of a positively charged amino acid in repeat III of voltage sensor segment S4. The dominance of the Wobbly mutation more closely resembles patterns of CACNA1A mutation in humans than previously described mouse recessive mutants (tottering, leaner, rolling Nagoya and rocker). Positive-charge neutralization in S4 has also been shown to underlie several cases of human dominant FHM1 with ataxia. The Wobbly mutant thus highlights the importance of the voltage sensor and provides a starting point to unravel the neuropathological mechanisms of this disease.

Keywords: Cerebellar ataxia, dominant mutation, ENU, genetic mapping, P/Q type calcium channel

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Voltage-dependent calcium channels (VDCCs) mediate the entry of Ca\(^{2+}\) into excitable cells and are also involved in a variety of Ca\(^{2+}\)-dependent cellular processes, such as muscle contraction, excitability, neurotransmitter release, gene expression, cell motility, cell division and cell death (Tsien & Tsien 1990; Tsien et al. 1991). In mammalian neurons, five major types of VDCCs, designated L, N, R, P/Q and T, have been identified based upon their pharmacological and electrophysiological properties (Bean 1989; Zhang et al. 1993). High-threshold VDCCs are multisubunit complexes consisting of \(\delta\), \(\alpha6\), \(\beta\), and \(\gamma\) subunits. Channel activity is mainly determined by the pore-forming and voltage-sensitive \(\alpha1\) subunit, which is encoded by a family of genes (\(\alpha1\) through \(\alpha1I\) and \(\alpha1S\)) (Birnbaumer et al. 1994; Lee et al. 1999). The auxiliary subunits \(\alpha6\), \(\beta\) and \(\gamma\) modulate channel activity (Ahlijanian et al. 1990; Campbell et al. 1988; Letts et al. 1998; Witcher et al. 1993). The Ca\(^{2+}\) channel \(\alpha1A\) subunit gene (Cacna1a) encodes the pore-forming protein of P/Q channels and gives rise to P/Q-type calcium currents. Cacna1a is highly expressed in the cerebellum, olfactory bulb, cerebral cortex, hippocampus, inferior colliculus and auditory brain stem (Fletcher et al. 1996; Tsumijimoto et al. 2002). The P-type VDCC is predominantly expressed in cerebellar Purkinje cells, whereas the Q-type is a prominent Ca\(^{2+}\) current in the cerebellar granule cells (Bourinet et al. 1999; Mintz et al. 1992; Zhang et al. 1993).

Missense mutations in the human CACNA1A gene underlie several neurological disorders, including episodic ataxia type 2, familial hemiplegic migraine 1 (FHM1) and spinocerebellar ataxia 6 (SCA6) (Ophoff et al. 1996; Yue et al. 1997). In mice, Cacna1a mutations have been identified in the cerebellar ataxic and dystonic mutants tottering (Cacna1a\(^{tg}\)), tgl, leaner (Cacna1a\(^{tg-\alpha3}\)), rolling Nagoya (Cacna1a\(^{tg-ro}\)) and rocker (Cacna1a\(^{Kr}\), rkt) (Doyle et al. 1997; Fletcher et al. 1996; Mori et al. 2000; Zwingman et al. 2001). In contrast to human CACNA1A dominant mutations, mice heterozygous for these mutations appear phenotypically normal. Only homozygous mutants exhibit varying degrees of cerebellar ataxia, absence seizures and paroxysmal dyskinesia, ranging from mild
Genetic mapping

Genomic DNA was extracted from tail tissue using a standard procedure. To localize the Wobbly mutation to a specific chromosomal region, a panel of 100 single-nucleotide polymorphisms (SNPs) between the B6 and C3H parental strains were used to scan the entire genome of 25 G2 Wobbly mice (14 affected, 11 normal) at a resolution of ~20 cm. High-throughput SNP genotyping by fluorescence polarization assay (Hsu et al. 1999; Kwon 2002) was undertaken using an Analyst HT fluorescence polarization reader (Molecular Devices, Sunnyvale, CA, USA). AlleleCall software (LJL Bio-systems, Sunnyvale, CA, USA) was used to refine the critical interval to 1.7 Mb in a total of 487 G3-G6 mice. For each SNP, a single-base extension (SBE) primer with a melting temperature of 60–80°C and length of 20–30 bases was designed so that its 3’ end annealed adjacent to the polymorphic base pair. Genomic DNA (10 ng) was amplified inside black, hard-shell, 96-well microplates (MJ Research, Waltham, MA, USA) in 5 μl reaction mixtures of 0.5 μl 10× polymerase chain reaction (PCR) Tag Gold buffer (Applied Biosystems, Foster City, CA, USA), 0.5 μl 25 mm MgCl2, 0.05 μl 2.5 mm deoxynucleotide triphosphates (dNTPs), 0.2 μl (1.25 μM each) PCR primers and 0.05 μl AmpliTaq Gold DNA polymerase (5 U/μl). Thermocycling conditions on a PTC-20225 DNA Engine Tetrad PCR machine (MJ Research) were as follows: denaturation at 95°C for 12 min, 15 cycles of 95°C for 30 seconds, 66°C for 30 seconds (step down 1°C per cycle) and 72°C for 30 seconds, followed by 35 cycles of 95°C for 30 seconds, 50°C for 30 seconds and 72°C for 30 seconds and a final extension of 72°C for 10 minutes. The PCR products were separated in 1.8% agarose gel by electrophoresis and visualized under UV light. The PCR products were purified with Wizard PCR Preps DNA Purification System (Promega, Madison, WI, USA) and labeled with [γ-32P]ATP using a random primer labeling kit (PerkinElmer). After the reaction was completed, the samples were loaded onto a 6% polyacrylamide gel and run at 100 V for 3.5 hours. The gel was then dried and exposed to X-ray film. The resulting autoradiographs were analyzed for the presence of bands corresponding to the markers.

Materials and methods

Mice and ENU mutagenesis

Male B6 mice (JAX Research Systems, Bar Harbor, MN, USA) received three intraperitoneal injections of ENU (85 mg/kg), as previously described (Fienniken et al. 2005). Ten weeks following the last ethynitrosourea (ENU) injection, the mutated males were mated to untreated female C3H mice (JAX Research Systems). To detect potential mutants, 3733 G1 progeny (C3HB6F1) of this mating were put through the same behavior and appearance screen as their founder male was bred to C3H females, and the resulting G2 progeny were used for motor behavior and appearance screening.

Wobbly mutation analysis

Total RNA was isolated from the brains of phenotypically homozygous mutant, heterozygous mutant and normal littermates. First-strand complementary DNA (cDNA) was synthesized by oligo(dT) priming (SuperScript First-Strand Synthesis System; Invitrogen, Burlington, ON, Canada). Reverse transcriptase–polymerase chain reaction (RT-PCR) primers were designed so that eleven 400- to 800-bp fragments were amplified. For genomic DNA from 93 G3-G6 mice (31 homozygous and 31 normal littermates) using the fluorescence polarization SNP genotyping assay: F: 5'-tctcagtgttgttgtatggagca-3', R: 5'-ctcgatgtgtgtgagggc-3' and SBE: 5'-ctccagcctctgcctgac-3'. For the SBE primer was added to the enzymatically treated PCR product. Each 10-μl reaction mixture was incubated at 94°C for 2 min, followed by 30 cycles of 94°C for 10 seconds and 55°C for 30 seconds.

Northern and Western blotting

For Northern blotting, 20 μg total RNA isolated from whole brain, skeletal muscle and spinal cord of 6-week-old mice were used for blot hybridization in ULTRAhyb hybridization solution (Ambion, Austin, TX, USA) with a 32P-labeled probe (RadPrime DNA labeling system; Invitrogen) consisting of nucleotides 427–1097 of the mouse Cacna1a transcript. For Western blotting, 25 μg protein extracted from cerebellum, forebrain, skeletal muscle and spinal cord of 6-week-old mice were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes and incubated...
with rabbit anti-Ca,2,1 (Cacna1a) polyclonal antibody (Chemicon, Temecula, CA, USA) in accordance with the vendor’s instructions.

Motor behavior tests
To assess the effects of the Wobbly mutation on coordinated motor behavior, 8-week-old Wb/Wb, Wb/w+, Wb/wtg-la, tg-la/+ and +/- littermates were subjected to the following tests of motor performance. 1 Hindlimb extension reflex: Mice were picked up and suspended vertically by the tail at an initial distance of 10 cm from a supportive surface for 3 seconds. The responses of each animal to 10 successive approaches to the support surface were then scored as follows: 0, no extension, clenching of limbs close to body; 1, hindlimb extended on physical contact with the surface, but not upon nose/washer contact; 2, hindlimb extension upon washer contact with surface; 3, hindlimb extension immediately prior to washer contact; 4, pre-emptive, vigorous extension of hindlimbs. Normal mice score 2–3 according to these criteria. 2 Four-limbs grip strength: A mouse grips a graspable surface rod (4-mm diameter), and the force required to relieve this grip is assessed using a dynamometer. Average scores (g) across the best four out of seven trials (30-second inter-trial periods) are reported. 3 Inclined platform: Mice are placed on a wire platform (1-×1-cm mesh), which is then inclined to 90°. The (time) seconds) it takes a mouse to traverse the entire distance of the platform (24-cm length) with gentle encouragement was then recorded. Mice that fall off the inclined platform are assigned a time of 120 seconds. 4 Beam cross: Mice are placed in the middle of a narrow, graspable runway (3 mm wide, 30 cm long, 28 cm high) and are examined for maneuverability between two stable platforms located at either end. Mice are scored as follows: 1, crosses easily without undue effort, can reverse on 3-mm edge, can reach platform from distance of 15 cm easily; 2, reaches platform with substantial difficulty, can reach platform from distances of <7 cm, tremor typically evident; 3, extreme difficulty in edge navigation, cannot make progress toward platform, but can maintain grasp on edge for a minimum of 8–10 seconds; 4, cannot maintain grasp on edge for 8–10 seconds. 5 Rotarod: Performance on a graspable rod (3-cm diameter) is assessed at several (constant) revolution speeds (rpm). Performance was scored as follows: 0 rpm, cannot maintain purchase on stationary rota rod; <8 rpm, cannot remain on rota rod at 8 rpm, typically such mice cannot grip rota rod in the inverted position; 15 rpm, maintains purchase on rota rod at 15 rpm for >5 cycles. Mice are typically examined at each speed for 3 min following a 2-day training period of 15 min each. On the test day, mice are examined for three trials of 3 min each and scored on the basis of their most successful trial.

Magnetic resonance imaging
A cohort of eight Wb/Wb, four Wb/w+ and five +/- littermates were used in two in vivo magnetic resonance imaging (MRI) scanning at 8 weeks of age. The whole-brain scan was done in parallel to produce 100-μm resolution three-dimensional (3D) data sets (Niemann et al. 2005) using a 7-tesla Varian MRI configured for multiple mice. The 3D images were initially aligned to one another by automated linear registration. Analyzing the Wb/Wb and Wb/+ groups separately, deformations were computed using nonlinear registration to bring the individual images in each comparison group into exact alignment. The spatial transformation bringing two brains into alignment was used as a quantitative measure of their difference in shape (Chen et al. 2006). The log Jacobians of these transformations were used to compute a Student’s t-statistic at every voxel.

Immunohistochemistry
Eight-week-old Wb/Wb, Wb/w+ and +/- littermates were lethally anesthetized with tribroethanol (Avertin, 250 mg/kg). Following a loss of deep tendon responses, the thoracic cavity was opened and mice were perfused through the left ventricle with 10 ml phosphate-buffered saline (PBS; 0.1 M phosphate buffer, pH 7.4; 0.9% NaCl), followed immediately by 4% paraformaldehyde in 0.1 M PBS (PFA) at 25°C. Brains were removed and postfixed overnight in PFA at 4°C. Following infiltration and wax processing, 7-μm paraffin sections were obtained through comparable structural levels for each genotype in either the sagittal or coronal plane. Following dewaxing and the quenching of endogenous peroxidase activity, tissue sections were incubated overnight at 4°C in either rabbit anti-Ca,2,1 (Cacna1a) or rabbit anti-tyrosine hydroxylase (TH) (Chemicon) diluted in primary media (PM, 5% goat serum, 0.2% Tween-20, 0.1 M PBS, pH 7.4). Following rinsing (3 × 5 min) in PM, sections were incubated for an additional 2 h in the appropriate biotin-labeled secondary antibody (1:200; Vector, Burlingame, CA, USA) at room temperature. After washing in 0.1 M PBS for 3 × 5 min, sections were incubated with streptavidin-horseradish peroxidase (HRP) for 1 h at room temperature, washed and developed using nickel-enhanced diaminobenzidine (DAB) (Vector).

Analysis of cerebellar molecular layer
Two sets of midsagittal cerebellar sections, spaced 200 μm apart, were prepared from three 8-week-old mice of each genotype (+/+), Wb/w+ and Wb/Wb. Segments of the cerebellar cortex between lobes V and VI were chosen for analysis. For each slice, linear segments of 600 μm were delineated on lobes V and VI. Thus, for each genotype, average areas of the molecular layer were determined from 36 segments (6 fields × 2 planes × 3 mice) of 600 μm in length. For each segment, areas were determined in μm² using COMIPS SIMPLE PCI image analysis software. Following an analysis of the normality of the distribution for each data set, genotypes were compared for significance using nonparametric t-tests.

Quantitative real-time PCR
Total cerebellar RNA was extracted from six Wb/Wb, six Wb/w+ and five +/- littermates at 8 weeks of age. The cDNA was prepared from 4 μg total RNA per reaction using SuperScript III First-Strand Synthesis System (Invitrogen) according to the manufacturer’s instructions. Oligo(dT) was used to prime the cDNA synthesis, and the reaction temperature was 50°C. Real-time PCR was performed in an Mx4000 Multiplex Quantitative PCR System (Stratagene, La Jolla, CA, USA) using Brilliant SYBR Green QPCR Core Reagent kits (Stratagene). Primers were 5'-agacctccctccagagtcac-3' and 5'-agtcagcaggagtcgtgctg-3' for Cacna1g (GenBank accession no. NM_007838); 5'-gaccttcgtgagagtctgta-3' and 5'-gctactcgagcagcaccctc-3' for Calb2 (GenBank accession no. NM_007586); 5'-cataggacacccacagc-3' and 5'-ggggagtgagctgctgctg-3' for Th (GenBank accession no. NM_003777); and 5'-gctactcgagcagcaccctc-3' and 5'-acagcaggcttgccgctg-3' for Gusb (GenBank accession no. NM_001608). DNA standards were PCR amplified, purified and quantified. Standard curves were created by preparing a dilution series of each standard for amplification. Each 25-μl reaction contained 1 μl template, 3.0 mM MgCl₂ and 400 nm each primer. The amplification protocol included a 10-min denaturation step, followed by 40 cycles of 95°C for 30 seconds and 59°C for 1 min. The dissociation curve was then generated by stepwise increases in temperature from 55°C to 95°C. Single PCR products were verified by observation of a single peak in the melting curve plot; analysis of variance was used to compare gene expression levels using SPS 11.0. Homogeneity of variance was tested using the Levene statistic. When variances were found to be equal (Cacna1g, P = 0.815; Calb2, P = 0.238), Fisher’s least-significant difference post hoc test was used. When Levene’s test was significant, indicating unequal variance (Th, P = 0.017), the Welch F-ratio was reported, and a conservative post hoc procedure (Tamhane’s 12 test) was used to identify genotype differences.

Results
Phenotypic screening of the Wobbly mutant
The founder Wobbly mutant was identified on the basis of its abnormal gait in a behavior and appearance screen of 3733
C3H6F1 hybrid progeny of ENU mutagenized C57BL/6J (B6) males and untreated C3H/HeJ (C3H) females. The Wobbly line was initially maintained by backcrossing to the C3H strain. Thirty of 64 (46.9%) G2 and G3 progeny displayed the abnormal gait of the founder mutant, indicating that the Wobbly phenotype is fully penetrant and inherited in an autosomal dominant manner. Heterozygous Wb+/ mice were recognizable at P21–28 by their unsteady gait, low body position, spayed hindlimbs and reduced locomotor activity. Heterozygotes had a normal body size and a life span similar to that of wild-type mice (data not shown). Of 187 intercross progeny of Wb+/ mutants, 48 (25.7%) mice displayed a severe neurological phenotype at P8, while 93 (49.7%) exhibited an unsteady gait by P21–28, and 46 (24.6%) appeared phenotypically normal. Thus, the unsteady gait of Wb+/ mutants was inherited as an autosomal semi-dominant trait. Homozygous Wb/Wb mice were easily identifiable at P8 by abnormal righting reflex and smaller size compared with their littermates. By P21, Wb/Wb mice developed severe ataxia and symptoms of dystonia and often lay on their sides with limbs extended in a stiff posture. Homozygotes died from starvation by P28 unless special provisions were made for access to food and water. Neither Wb/+ nor Wb/Wb mice displayed paroxysmal dyskinesia or motor seizures, nor did abnormal motor behavior progressively worsen with age.

Genetic mapping of the Wobbly mutation
A genome scan of 14 affected and 11 unaffected G2 progeny of the founder Wobbly G1 male and C3H females revealed that the characteristic unsteady gait cosegregated with SNP marker ss4319508 at 82.9 Mb on chromosome (Chr) 8. An extensive set of SNP markers was used to genotype 497 G3-G6 backcross and intercross progeny to map the mutation to a 1.7-Mb interval between SNP markers ss5034585 (82.7 Mb) and ss5109919 (84.4 Mb) (Fig. 1). Cacna1a was the most appealing candidate gene within this interval.

Wobbly has a missense mutation in Cacna1a
Sequencing of the 6.5-kb coding region of Cacna1a cDNA (GenBank accession no. U76716) from Wobbly mutants and normal littermates revealed a G-to-T transversion at nucleotide 3764, leading to an arginine (R) 1255 to leucine (L) substitution in Wb/Wb and Wb+/+ mice (Fig. 1). This missense mutation was located in exon 27 of the murine Cacna1a gene and was not found in genomic DNA from the B6 and C3H parental strains. Segregation analysis of the point mutation, using a fluorescence polarization genotyping assay, revealed coinheritance of the missense mutation and ataxic phenotype in all 31 Wb+/Wb and 31 Wb+/+ mice tested, while all 31 normal littermates were wild type at the Wobbly locus. R1255 is a highly conserved, positively charged amino acid located in the middle of the repeat III S4 transmembrane segment (Fig. 1). The S4 segments of the calcium channel represent the voltage sensor and are characterized by a series of positively charged amino acids at every third position. Northern and Western blot analyses did not detect any differences in brain Cacna1a mRNA and protein expression between Wb/Wb, Wb/+ and +/+ littermates (Fig. 2).

Wobbly and leaner mutations are allelic
To test for complementarity to a known Cacna1a allele such as leaner, Wb/þ were crossed to tg-la+/ mice. As Wb/þ mice display a mild ataxic phenotype, while tg-la/þ mice are phenotypically normal, it was expected that compound heterozygotes (Wb/tg-la) would exhibit an ataxic phenotype similar to Wb/Wb mice if these mutants were allelic to one another. In contrast, if Wobbly and leaner represented complementary loci, compound heterozygotes should exhibit a phenotype similar to that seen in Wb/þ mice. Among 85 progeny of this cross, 21 exhibited a severe dystonic phenotype similar to that seen in Wb/Wb mice, 20 displayed an ataxic phenotype resembling Wb/+ and 44 mice were phenotypically normal. The SNP genotyping of both alleles revealed that all severely affected mice were heterozygous for both the leaner and Wobbly mutations, whereas all the mildly ataxic mice were heterozygous for the Wobbly mutation only. The phenotypically normal mice were either +/+ or tg-la/+. This noncomplementation with leaner is consistent with Wobbly being an allele of Cacna1a.

Motor coordination deficits in Wobbly mutant mice
In hindlimb extension reflex tests, Wb/Wb and Wb/tg-la mice exhibited an abnormal posture characterized by spasmodic movements and flexion of the hindlimbs. Wb/+ mice also had a deficit in this test, while no significant difference was observed between tg-la/þ and +/+ littermates (Fig. 3). Wb/Wb and Wb/tg-la mice both exhibited significantly reduced grip strength compared with +/+ mice. No significant differences in grip strength were observed between Wb/þ, tg-la/þ and +/+ mice (Fig. 3). In inclined-plane performance, Wb/Wb and Wb/tg-la mice performed poorly. Wb/þ mice required a significantly longer time than +/+ mice to traverse the inclined platform. Wild-type controls and tg-la/þ mice were indistinguishable in this test (Fig. 3). In the narrow-beam cross, a test of fine motor control, both Wb/Wb and Wb/tg-la mice were unable to cross the beam toward the platform. Wb/þ mice showed some reduced maneuverability compared with wild type and tg-la/þ mice (Fig. 3). In rotarod performance, both Wb/Wb and Wb/tg-la mice were not able to maintain purchase on a stationary rod. Wb/þ mice exhibited substantial difficulty at even low rotation speeds compared with +/+ and tg-la/þ mice (Fig. 3). In each of the motor coordination tests, Wb/Wb exhibited significant deficits compared with Wb/þ mice (P values in Table 1).

Cerebellar atrophy and reduction of the molecular layer in Wobbly mutant mice
The MRI of the brain and skull in Wb/Wb and Wb/þ mice showed significant cerebellar atrophy compared with +/+ mice. These differences were located primarily in the superior and medial regions of the cerebellum, including lobes III and V (Fig. 4). Segmentation measurement confirmed reduced
cerebellum-to-brain volume ratios of 7% and 17% in Wb/þ and Wb/Wb, respectively (P < 0.01), compared with +/+ mice. Analysis of the overall cytoarchitecture of +/+, Wb/þ and Wb/Wb adult brain stained with 0.2% cresyl violet revealed no gross cytological abnormalities (data not shown). However, measurements of the width and total area of the molecular layer of the cerebellum revealed significant reduction in Wb/þ and Wb/Wb compared with +/+ mice (Fig. 5). Neither Purkinje cell loss nor a decrease in the size of the granule cell layer was apparent in thionin-stained cerebellar sections from Wb/þ and Wb/Wb mice (Fig. 5). No significant differences were observed in the position or intensity of specific anti-Cacna1a staining within the cerebellum and hippocampus of +/+, Wb/þ and Wb/Wb mice (data not shown).

Figure 1: Positional cloning of the Wobbly mutation. (a) High-resolution haplotype mapping of Wobbly mutation on mouse Chr 8. White squares indicate C3H homozygotes; black squares indicate B6 homozygotes; gray squares indicate B6/C3H heterozygotes. The SNPs defining the minimal interval are shown in bold text. Map positions are in accordance with the public mouse genome assembly (www.ensembl.org/mus_musculus). (b) The cDNA sequencing of mouse Cacna1a revealed a G-to-T substitution at nucleotide residue 3764, leading to an arginine (R) to leucine (L) change at residue 1255 in Wb/Wb and Wb/þ mice. (c) Protein sequence alignment of the repeat III voltage sensor S4 segment (bold text) of the P/Q-type Ca²⁺ channel across various species. The Wobbly mutation R1255L is positioned at the fourth positively charged amino acid (underlined text).
Altered cerebellar mRNA expression of Th, Calb2 and Cacna1g genes in Wobbly mutant mice

Previous studies have shown that the expression of Th, Calb2 and Cacna1g genes is altered in the cerebellum of tg-la/tg-la mutant mice (Hess & Wilson 1991; Nahm et al. 2002, 2005). Therefore, we investigated the expression of these genes in the cerebellum of Wobbly mice. In Wobbly mutant mice, there was a significant effect of genotype on expression levels of Th, encoding calbindin 2 (also known as calretinin) (F(2,14) = 6.27, P = 0.011) and of Cacna1g, encoding the alpha 1G subunit of the T-type VDCCs (F(2,14) = 8.75, P = 0.003), when normalized by expression of the Gusb housekeeping gene (Fig. 6). Gusb expression levels were not significantly different between genotypes (F(2,14) = 1.708, P = 0.22). Real-time PCR standard curve slopes ranged from 3.2 to 3.5, indicating ~93-105% amplification efficiencies. The TH immunostaining localized the increased expression of Th within adult Wb/Wb cerebellum to a proportion of the Purkinje neurons (data not shown).

Discussion

Cerebellar ataxia was the predominant neurological phenotype observed in Wobbly mice. The mild cerebellar ataxia exhibited by Wb/+ mice at P21–28 resembled the motor phenotype described for homozygous tottering (tg/tg) mice (Green & Sidman 1962; Nakane 1976). The early onset (P8) of severe motor abnormalities and premature death of unaided Wb/Wb mice resembled the more severe phenotype described for homozygous leaner (tg-la/tg-la) mice (Meier & MacPike 1971). To address whether heterozygotes for other Cacna1a mutant alleles may have previously undetected motor deficits, we used the severe Cacna1a mutation leaner as a phenotypic comparative control in our battery of motor coordination tests. These tests clearly showed that Wb/+ mice have significant motor deficits without loss of muscle strength. In contrast, tg-la/+ and +/+ mice were indistinguishable in these tests. Wb/Wb and Wb/tg-la mice showed severe motor coordination deficits and muscle weakness compared with Wb/+, tg-la/+ and +/+ mice. These results show that the Wobbly mutation is semidominant, whereas leaner is recessive.

Analysis by MRI revealed significant contraction of the cerebellum in Wb/Wb and Wb/+ mice. Measurements of the width and total area of the molecular layer indicated significant reduction in Wobbly mutants, which is similar to the molecular layer-specific decrease in cerebellar volume reported for tg/tg mice (Isaacs & Abbott 1995). Cerebellar atrophy was more severe in Wb/Wb than in the Wb/+ mice, which correlated well with the degrees of cerebellar ataxia exhibited by these genotypes. Although motor function in Wb/Wb mice was as severely affected as that reported for tg-la/tg-la mice, Wb/Wb showed no signs of the cerebellar Purkinje cell loss reported for tg-la/tg-la mice (Herrup & Wiczyński 1982). Cacna1a immunostaining and Northern and Western blot analyses showed no obvious change in Cacna1a gene expression within the cerebellum and hippocampus of Wb/Wb and Wb/+ mice. These findings suggest that the neuropathological alterations of Wobbly mutants are the result of post-translational impairment of Ca^{2+} channel activity, rather than progressive apoptotic neuron death in the cerebellum.

The point mutation identified in Wobbly mutants is very close to the rolling Nagoya (tg-rol) mutation in its structural location within the P/O-type Ca^{2+} channel. Both mutations are located in the voltage sensor of the repeat III S4 segment.
The Wobbly mutation leads to the positive charge-neutralizing substitution R1255L, and the rolling Nagoya mutation leads to the positive charge-neutralizing change R1262G (Mori et al. 2000) (Fig. 1). Leucine (L) and glycine (G) belong to the aliphatic R group, which is nonpolar and hydrophobic. Although these mutations lead to similar ataxic phenotypes in the homozygous state, Wb/Wb mice have a more severe cerebellar ataxia with an earlier age of onset than described for tg-la/+ mice (Nakane 1976; Oda 1973). Notably, Wobbly is a semidominant mutation, whereas rolling Nagoya is recessive.

Transmembrane S4 segments have been implicated as voltage sensors of different voltage-gated channels (Garcia et al. 1997; Liman et al. 1991; Pathak et al. 2005; Stuhmer et al. 1989) and have a characteristic arrangement with a varying number of positively charged amino acids at every third position. It has been shown that the rolling Nagoya mutation produces a deficit in the gating properties of the P-type Ca\textsuperscript{2+} channel in Purkinje cells and in transfected BHK6 cells transiently expressing the rolling Nagoya mutant P/Q-type channel (Mori et al. 2000). Functional and structural studies of the voltage-gated potassium channel Shaker (Kv) suggest that the first four positively charged residues (counted from the extracellular side of S4) are the most relevant for voltage sensing (Aggarwal & MacKinnon 1996; Jiang et al. 2003; Seoh et al. 1996). The Wobbly mutation leads to neutralization...
of the fourth positively charged arginine, and its position complies with the characteristic arrangement of the positively charged amino acids at every third position. However, the rolling Nagoya mutation leads to the neutralization of the seventh positively charged arginine, which is an extra positively charged residue in repeat III S4 in a position that deviates from the characteristic arrangement of the positively charged amino acids at every third position (Figs 1, 7). Therefore, the Wobbly mutation likely alters a functionally more important positively charged residue in S4 than that altered by rolling Nagoya, and possibly induces a greater impairment in gate charging of the P/Q-type Ca$^{2+}$ channel than the rolling Nagoya mutation does. This could explain why Wobbly leads to more severe cerebellar ataxia and is a semidominant mutation.

Real-time PCR analysis revealed significantly enhanced Th expression in the cerebellum of both Wb/+ and Wb/Wb mice at 8 weeks of age, which was confirmed by TH immunostaining in adult Wb/Wb cerebellum. Previous studies have shown increased expression of TH in cerebellar Purkinje cells of adult tg/tg, tg-la/tg-la and tg-rol/tg-rol mice, while adult heterozygotes for these mutations had no significant change in TH expression (Austin et al. 1992; Hess & Wilson 1991; Sawada & Fukui 2001; Sawada et al. 2004). The abnormally increased TH in Purkinje cells of tg-la/tg-la mice was not enzymatically active and not associated with catecholamine synthesis (Sawada et al. 2004). The transcription of Th is regulated by Ca$^{2+}$ (Nagamoto-Combs et al. 1997) and is facilitated by the activation of N-type or L-type Ca$^{2+}$ channels (Brosenitsch & Katz 2001). In tg/tg mice, L-type Ca$^{2+}$ channel expression in Figure 4: The MRI scans of Wobbly mouse brain. Upper panels: Average Wb/Wb (homozygous ‘Wobbly’), Wb/+ (heterozygous ‘Wobbly’) and +/- (control) images are shown in the horizontal and sagittal orientation. Note the reduced size of the cerebellum in the Wb/Wb and Wb/+ mice. Lower panels: Average Jacobian data are overlaid to indicate regions and severity of abnormalities where $P < 0.05$. White contour lines indicate $P < 0.002$ (5% false discovery rate in Wb/Wb mice). Bar chart: Differences in cerebellum–brain volume ratios are shown (*$P < 0.01$).
Purkinje cells was increased to compensate for the altered function of the P/Q-type channel (Campbell & Hess 1999), and chronic injections of an L-type Ca$^{2+}$ channel blocker decreased Th mRNA expression in the cerebellum (Fureman et al. 2003). Thus, the enhanced expression of Th in the Purkinje cells of Wb/+ mice may reflect neural dysfunction caused by alteration of the intracellular Ca$^{2+}$ concentration that may induce the dominant ataxia of these mice.

Expression of Calb2 was significantly decreased in the cerebellum of Wb/Wb and Wb/+ mice. Decreased expression of Calb2 has also been observed in the cerebellum of tg-la/tg-la and tg/tg mice (Cicale et al. 2002; Nahm et al. 2002), while there was no change in Calb2 expression in tgl/+ mice (Cicale et al. 2002). Calb2 is one component of the extensive Ca$^{2+}$ buffering system that protects cells against an abrupt increase in intracellular Ca$^{2+}$ concentration (Baimbridge et al. 1992). Calb2 null mutants exhibit impaired motor coordination without major disturbance of the cerebellar cytoarchitecture (Schiffmann et al. 1999). It has been hypothesized that impaired Ca$^{2+}$ homeostasis in parallel fiber/Purkinje cell synapses could deteriorate cerebellar control of motor coordination, resulting in the cerebellar ataxia of tgl/tg-la and Calb2 null mice (Cheron et al. 2000; Dove et al. 2000; Nahm et al. 2002). The enhanced expression of Th in Wb/+ mice could indicate altered intracellular Ca$^{2+}$ concentration in the Purkinje cells, while the decreased expression of Calb2 in the cerebellum suggests a possible deficit in balancing the altered intracellular Ca$^{2+}$ concentration. Thus, it is tempting to speculate that altered intracellular Ca$^{2+}$ concentration and impaired Ca$^{2+}$ homeostasis may contribute to the motor coordination deficits of Wb/+ mice. Consistent with this hypothesis, tgl/+ mice have unchanged levels of cerebellar TH and Calb2, and exhibit normal neurological behavior (Austin et al. 1992; Cicale et al. 2002).

Cerebellar expression of Cacna1g was significantly decreased in both Wb/+ and Wb/Wb mice. Cacna1g expression is also significantly decreased in whole cerebellum and granule cells of tg-la/tg-la mice (Nahm et al. 2005). However, tg-la/tg-la Purkinje cells showed increased Cacna1g expression. It has been speculated that the paroxysmal dyskinesia exhibited by tg-la/tg-la mice is related to this increase in Purkinje cell Cacna1g expression (Nahm et al. 2005). If this hypothesis were correct, the lack of paroxysmal dyskinesia in Wobbly mice would be predictive of normal Cacna1g expression in Wobbly Purkinje cells.

In the human CACNA1A gene, seven missense mutations leading to neutralization of the positive charge in voltage sensor S4 segments have been identified (Fig. 7), six of which were identified in FHM1 families with or without cerebellar ataxia (Alonso et al. 2004; Ducros et al. 2001; Friend et al. 1999; Ophoff et al. 1998). The seventh mutation

Figure 5: Thionin staining of the cerebellum of (a) +/+ , (b) Wb/+ and (c) Wb/Wb mice. The difference in molecular layer area is obvious. Neither Purkinje cell loss nor decrease in the size of the granule cell layers was apparent. (d) Average area (μm$^2$) of the cerebellar molecular layer of +/+ , Wb/+ and Wb/Wb mice calculated from 36 segments of 600 μm in length. Molecular layer area was significantly reduced in Wb/+ and Wb/Wb mice (**P < 0.001). Scale bar indicates 100 μm.

Figure 6: Quantitative real-time PCR analysis of Cacna1g, Calb2 and Th gene expression in the cerebellum of Wb/Wb, Wb/+ and +/+ mice. Cacna1g expression was significantly lower in Wb/+ (P = 0.019) and Wb/Wb (P = 0.001) mice than in +/+ mice. Calb2 expression was significantly lower in Wb/+ (P = 0.033) and Wb/Wb (P = 0.004) mice than in +/+ mice. Th expression was significantly greater in Wb/+ (P = 0.005) and Wb/Wb (P = 0.001) mice than in +/+ mice. Expression levels of Cacna1g, Calb2 and Th were not significantly different between Wb/+ and Wb/Wb mice (**P < 0.05).
was associated with an unconfirmed diagnosis of SCA6 in a 3-year-old patient, in which migraine could not be excluded because of the patient’s young age (Tonelli et al. 2006). A knockin mouse model carrying the human FHMI R192Q mutation showed a gain-of-function effect on neuronal Ca2+ current, enhanced neurotransmitter release and lowered threshold for cortical spreading depression (van den Maagdenberg et al. 2004). However, the R192Q mutant mouse appeared phenotypically normal and had no cytoarchitectural abnormalities in the brain (van den Maagdenberg et al. 2004). In FHMI patients with ataxia who have been examined by MRI, cerebellar atrophy was detected (Alonso et al. 2004; Ducros et al. 2001; Friend et al. 1999). Interestingly, the mouse Wobbly mutation leading to neutralization of the fourth positively charged amino acid in repeat III S4 is remarkably similar to a human missense mutation leading to neutralization of the second positively charged amino acid (R1347Q) in repeat III S4 in a Portuguese FHMI family with cerebellar ataxia and atrophy (Figs 1,7) (Alonso repeat III S4 in a Portuguese FHMI family with cerebellar ataxia and atrophy (Figs 1,7) (Alonso et al. 2001)). The position of the Wobbly (Wb), tottering (tg), rolling Nagoya (tg-roll), leaner (tg-la) and rocker (trk) mutations of mouse Cacna1a are indicated (gray triangles). The human neurological diseases caused by mutations in the voltage sensor S4 segments of human CACNA1A are named (boxes, top). The positions of the human mutations (closed circles) are according to GenBank accession no. X99897. FHMI/A; FHM1 with cerebellar ataxia.

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