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Mutations in the *Cacnl1a4* calcium channel gene are associated with seizures, cerebellar degeneration, and ataxia in tottering and leaner mutant mice

Johannah Doyle,[1,2] Xiaojia Ren,[1] Greg Lennon,[3] and Lisa Stubbs[1]

[1]Biology Division, Oak Ridge National Laboratory, P.O. Box 2009, Oak Ridge, Tennessee 37831-8077, USA
[2]University of Tennessee-Oak Ridge School of Biomedical Sciences, Oak Ridge National Laboratory, P.O. Box 2009, Oak Ridge, Tennessee 37831-8077, USA
[3]Human Genome Center, Lawrence Livermore National Laboratory, 7000 East Ave., L-452, Livermore, California 94551, USA

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Abstract. Tottering and leaner, two mutations of the mouse tottering locus, have been studied extensively as models for human epilepsy. Here we describe the isolation, mapping, and expression analysis of *Cacnl1a4*, a gene encoding the alpha subunit of a proposed P-type calcium channel, and also report the physical mapping and expression patterns of the orthologous human gene. DNA sequencing and gene expression data demonstrate that *Cacnl1a4* mutations are the primary cause of seizures and ataxia in tottering and leaner mutant mice, and suggest that tottering locus mutations and human diseases, episodic ataxia 2 and familial hemiplegic migraine, represent mutations in mouse and human versions of the same channel-encoding gene.

Introduction

Tottering (*tg*) and more recently defined mutations, leaner (tg^{la}) and rolling mouse Nagoya (tg^{rol}), have been studied extensively as models for human epilepsy (reviewed by Kostopoulos 1992). Although they are clearly allelic (Tsuji and Meier 1971), animals homozygous for the recessively inherited *tg*, tg^{la}, and tg^{rol} mutations exhibit different types of seizures and develop cerebellar ataxia with widely varying degrees of severity. Absence seizures, manifested by sudden arrest of movement, fixed staring gaze, and isolated myoclonic jerks in mice, are associated with each of the three mutations and coincide with an electrocorticogram "spike-and-wave" pattern similar to that observed in patients with human petit mal epilepsy (Noebels and Sidman 1979). Interestingly, although roller and leaner mutants exhibit markedly more severe forms of ataxia, only the milder *tg* mutation produces seizures of the focal motor type (Noebels and Sidman 1979; Green and Sidman 1962). Symptoms appear in *tg/tg* mice 3–4 weeks after birth, with seizures decreasing in both frequency and intensity as the animals age (Meier and MacPike 1971). Leaner mice, by contrast, display first signs of ataxia as early as 8–10 postnatal days, and most affected animals die around the time of weaning (Sidman et al. 1965).

The anterior cerebellum of the leaner mouse is noticeably reduced in size when compared with that of normal littermates. Pyknotic granule cells are present in leaner mutants as early as 10 postnatal days, with massive degeneration of granule, Purkinje, and Golgi cells at a later stage (Sidman et al. 1965; Herrup and Wilcynski 1995). Atrophy of specific inferior olive neurons has also been observed in leaner mice (Heckroth and Abbott 1996). Milder granule and Purkinje cell abnormalities have also been observed in brains of adult *tg/tg* animals (Meier and MacPike 1971), which also display an anomalous proliferation of noradrenergic terminals arising from the brainstem locus ceruleus (Levitt and Noebels 1981). The complex array of phenotypes arising from mutations at this single locus and their significance to studies of human neurological disease have added a special element of interest to the search for the tottering gene. Tottering is located in central mouse Chromosome (Chr) 8 (Yoon 1969; Tsuji and Meier 1971), in a region bracketed by intervals of genomic homology to human Chr 19p13.1 and 16q12 (Ceci 1994). Although the location of the human homolog was therefore uncertain, a gene with excellent potential as a *tg* candidate has recently been assigned to human 19p (Margolis et al. 1995; Diriong et al. 1995). This gene, CACNL1A4, which encodes the alpha subunit of a proposed P-type calcium channel, is closely related to rabbit and rat genes that are expressed at high levels in the cerebellum and widely throughout the brain (Mori et al. 1991; Starr et al. 1991).

Here we describe the isolation of mouse *Cacnl1a4* cDNA sequences, assignment of the gene to the *tg* region of mouse Chr 8, and localization of orthologous human sequences within the physical map of Chr 19p13.1. *Cacnl1a4* transcripts can be detected as early as 10.5 days of prenatal development in normal mice, but are present in anomalously low levels in the brains of leaner mutant animals. Our data demonstrate that absence of one of two major alternative *Cacnl1a4* transcripts, probably owing to defective splicing, is the primary cause of absence seizures, cerebellar degeneration, and ataxia in leaner mutant mice, while a point mutation in sequences encoding a conserved membrane-spanning channel domain gives rise to focal motor seizures in tottering animals. Finally, we have mapped the orthologous human CACNL1A4 gene to a region flanked by markers D19S221 and D19S226 in human Chr 19p13.1. The results of these studies implicate the human CACNL1A4 gene as a causative factor in human Chr 19p-linked diseases episodic ataxia, type 2 (EA2; Kramer et al. 1994) and familial hemiplegic migraine (MHP; Joutel et al. 1993), and indicate that tottering mutants may represent mouse models for both human diseases.

Materials and methods

Mouse genetic mapping. For initial studies, a 372-bp *Cacnl1a4* gene fragment was generated from mouse brain cDNA with primers derived from the published rat gene *rbA* gene sequence (Starr et al. 1991; 5′ACCTCAGTACCATCTCTGAT3′; 5′GTGTCCGGCCGCTCCTGTGC3′) at an annealing temperature of 60°C, for 30 cycles in a GeneAmp 9600 machine (Perkin-Elmer/Cetus). The mouse *Peg3* probe was PCR generated with published primer sequences (Kuroiwa et al., 1996) with 100 ng of mouse genomic DNA as template and an annealing temperature of 65°C for 30

Correspondence to L. Stubbs

cycles. The human *Lpl* cDNA clone (Hua et al. 1991) was obtained from the American Type Culture Collection (ATCC); *Phkb* was represented by a human cDNA clone (identified by EST206665) purchased from Research Genetics, Inc. Human β-actin was provided with human Northern blots by Clonetech, and the *Es22* cDNA clone (Ovnic et al. 1991) was kindly donated by Richard Swank. All probes were radiolabeled and hybridized as previously described (Stubbs et al. 1990). The 8-kb mouse cDNA clone isolated with this probe (see below) was mapped by use of a previously described *Mus musculus* × *M. spretus* interspecific backcross (Doyle et al. 1996a, 1996b; Stubbs et al. 1996). Mapping data for *Junb, Jund, Adcy7,* and *Rfx1* were taken from these published studies; information regarding those probes can be obtained from the original references. Backcross mapping results were compared with a more limited set of data generated on a *Mus musculus* × *M. castaneus* intercross segregating the tg^{la} phenotype. To create this intercross, female animals, heterozygous for the closely linked *Os* and tg^{la} mutations (*Os* +/+ tg^{la}, animals obtained from The Jackson Laboratory, Bar Harbor, Maine) were crossed with *M. castaneus* males, and offspring with normal digits (carrying the tg^{la} allele in heterozygous form; Green and Sidman 1962) were intercrossed. DNA was prepared from offspring displaying the leaner phenotype, digested with restriction enzymes, transferred to nylon membranes, and hybridized as previously described (Stubbs et al. 1990).

Human physical mapping. To localize genes within the established physical map of human Chr 19 (Ashworth et al. 1995), we hybridized radiolabeled probes to high-density filter arrays of Chr 19-specific cosmids. The cosmid clones present in this library have been fingerprinted and assembled into contigs, assigned to specific positions on the Chr 19 physical map by high-resolution FISH mapping techniques, and restriction mapped with semi-automated methods, as described in previously published reports (Ashworth et al. 1995 and citations therein). *Eco*RI-digested cosmid DNA samples were electrophoresed, transferred to nylon membranes, and hybridized essentially as described (Stubbs et al. 1990).

RNA isolation and analysis. To identify leaner homozygotes before the age of onset of symptoms, we used a cross involving the tightly linked *Os* mutation, similar to that described above. Doubly heterozygous mice (*Os* +/+ tg^{la}) were intercrossed, and brains of animals with normal digits (+ tg^{la}/+ tg^{la}) were snap-frozen in liquid nitrogen. RNA was prepared and polyA selected with commercial kits (Rapid total RNA isolation kit and Mini-Oligo(dT) cellulose spun columns; 5 Prime 3 Prime, Inc., Boulder, CO). RNA (2.5 μg per lane) was electrophoresed through 0.8% agarose gels containing 19% formaldehyde. After electrophoresis, the gels were soaked for 30 min in 50 mM NaOH, neutralized briefly in 0.1 M Tris-HCl, and blotted to Duralon nylon membranes (Stratagene) in 20× SSC. Blots were rinsed in 2× SSC and dried 2 h under vacuum at 80°C, and UV crosslinked in Stratalinker chamber (Stratagene) before hybridization.

Human brain expression was investigated by hybridizing a commercially prepared Northern blot (Clonetech) with a 3-kb *Eco*RI fragment of the mouse cDNA clone (see below). Hybridizations were performed as described above, except that hybridization buffer contained 45% formamide (versus 50%), and washing buffer contained 1X SSC.

cDNA library construction and clone analysis. To isolate mouse *Cacnl1a4* sequences, a cDNA library was constructed with 4 μg of polyA+ RNA derived from cerebella of 12-day-old C3Hf inbred mice. Double-stranded, oligo-dT-primed cDNA was generated with a commercial kit (Amersham) and size selected on a 10–40% sucrose gradient spun in an SW41 rotor at 26,000 rpm, 24 h at 20°C. Fractions were collected (0.5 ml) and the DNA precipitated with ethanol before being combined into three pools containing fragments of 0.5–2 kb, 2–4 kb, and >4 kb in length, respectively. cDNA pools were ligated to *Eco*RI-*Sma*I linkers, and after removal of excess linkers by column chromatography (Size-Sep 400 columns, Pharmacia), each pool was ligated to *Eco*RI-cut lambda ZapII vector (Strategene) and plated as separate sub-libraries. Positive clones were isolated from the largest pool by hybridization of plaque lifts (using Duralon nylon filters; Strategene) with the radioactively labeled rat PCR probe. Phage clones were converted to plasmid form, according to manufacturer's instructions, before further analysis.

DNA sequence analysis. PCR products corresponding to tottering and DBA/2 membrane-spanning domains I–IV were generated by RT-PCR from brain RNA samples, with primers derived from related regions of the rat gene sequence (Starr et al. 1991) as follows. Domain I: 5′ TCCCAAGTTTACAAGTCGTC, and 5′ GGTTCAGCATAAAAAAGGAG (nucleotide position 633-1068 in rat sequence; 435-bp product); Domain II: 5′ ATTGTCCACTACAACCAGCC, and 5′ AGGTTGTCCACCGCGATAGC (pos. 1523-2156; 633-bp product); Domain III: 5′ AAAGGAAAGGACATCAACAC, and 5′ AAGGCCACAAAGATATTGAC (pos. 3857-4379; 522-bp product); and Domain IV: 5′ TGGGAATAACTTCATCAACC, and 5′ ACAGCAACAAAGAGATTCAG (pos. 4804-5277; 473-bp product). PCR products corresponding to tottering and DBA/2 conserved domains were purified using the Qia-quick gel extraction kit (Qiagen). Purified PCR products were analyzed with the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction kit and reactions analyzed on an ABI 373 automated sequencer, according to manufacturer's instructions. Several independent samples from each region of the normal and mutant mice were sequenced from both forward and reverse primers for verification. Sequence was also generated manually to confirm these results, by use of radioactive primers and reagents supplied with a commercial kit (fmol DNA sequencing system, Promega), according to manufacturer's instructions. Manual sequencing reactions were analyzed on a 6% acrylamide sequencing gel and exposed to X-ray film before examination.

Results

Mouse genetic mapping. Although the DNA sequence of CACNL1A4 was not available at the outset of this study, portions of the 19p-linked human α_{1A} calcium channel gene had been isolated in a previous study (Margolis et al. 1995). The sequence of this partial human cDNA clone was shown to be strikingly similar to 3′-portions of published full-length rat and rabbit brain α_{1A} calcium channel gene sequences (Mori et al. 1991; Starr et al. 1991). To investigate a possible connection between *Cacnl1a4* and tottering mutations, we therefore used reverse transcript-polymerase chain reaction (RT-PCR; Veres et al. 1987) with primers selected from the published rat *rbA* gene sequence to generate a probe from mouse brain cDNA. Analysis of the resulting 372-bp product revealed 97% nucleotide sequence identity with the rat sequence (nucleotides 6188–6560), and 87% identity with the related portion of the human CCA54 cDNA fragment (Margolis et al. 1995; not shown). The chromosomal position of the mouse gene was determined with a 160-member *Mus musculus* × *M. spretus* interspecific backcross (IB) previously used to map several genes from the *tg* region. In addition to *Jund, Junb, Ucp, Rfx1,* and *Adcy7,* whose segregation patterns in this cross have already been reported (Doyle et al. 1996a, 1996b), we mapped several additional markers to aid in the correlation of these data with a more limited set obtained from a *Mus musculus* × *M. castaneus* interspecific intercross segregating the tg^{la} allele. These studies localized the mouse α_{1A} calcium channel gene sequence clearly into the *tg* interval of mouse Chr 8 (Fig. 1).

Mouse cDNA isolation and gene expression studies.

Developmental profile of mouse gene expression. Published reports have indicated that both rabbit and rat α_{1A} calcium channel genes are expressed at highest levels in cerebellum, with lower levels of transcription detected in other brain regions and in spinal cord, heart, and kidney (Mori et al. 1991; Starr et al. 1991). The human gene probe has also been used to demonstrate expression in primate cerebellum, but other sites of transcription have not been tested (Margolis et al. 1995). Since tg^{la} homozygotes show first signs of ataxia and seizures at an early age, we first examined the developmental profile of mouse *Cacnl1a4* gene expression in normal mice. We used the 372-bp RT-PCR probe to isolate clones from a cDNA library representing size-selected transcripts from 12-day-old mouse cerebellum. Sequence analysis of a 300-bp segment extending from the 5′-terminus of the longest mouse cDNA

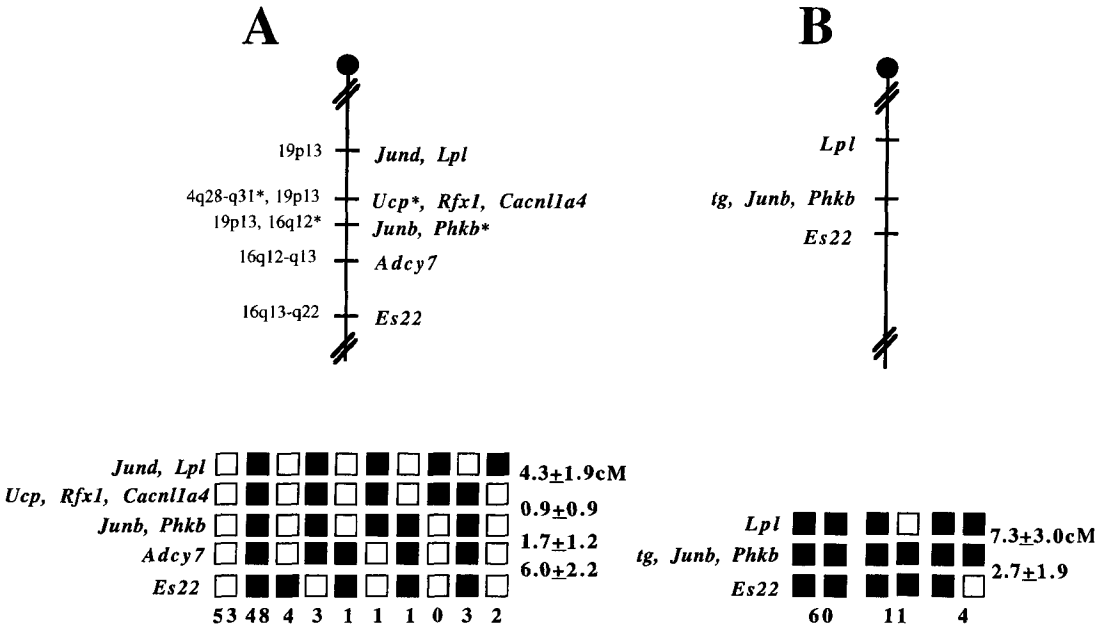


Fig. 1. Assignment of *Cacnl1a4* to mouse Chr 8. **(A) Top:** Partial map of Chr 8 as calculated with a *Mus musculus* × *M. spretus* interspecific backcross. Chromosomal locations of orthologous human genes are shown at far left; where more than one human linkage group is represented by co-segregating mouse genes, asterisks have been placed next to gene symbols and corresponding human map positions. **Bottom:** Summary of the segregation patterns of *Cacnl1a4* and flanking genes in 116 interspecific backcross (IB) animals. Each column represents the chromosome inherited by groups of IB progeny from their C3Hf × *M. spretus* F_1 parent, with black boxes representing C3Hf alleles and white boxes denoting *M. spretus* alleles for a given locus. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. Recombination distances between loci (in centimorgans) with standard error at the 95% confidence limit were calculated with Map Manager software (Manly 1993) and are indicated to the right and between each pair of rows, representing distribution patterns for adjacent sets of genes. Variant restriction fragments used to follow the segregation of each gene were as follows: Segregation of *Cacnl1a4* was traced with variant *Taq*I fragments [*M. musculus* (M) = 4.8 kb; *M. spretus* (S) = 3.5 kb, 1.5 kb]. *Lpl* with *Eco*RI [(M) = 3.0 kb; (S) = 4.0 kb], *Phkb* with *Eco*RV [(M) = 7.5 kb; (S) = 3.4 kb, 2.7 kb], and *Es22* with *Eco*RV [(M) = 3.0 kb; (S) = 2.0 kb]. Probes and variant fragments used to map *Jund, Ucp, Junb, Rfx1,* and *Adcy7* have been described elsewhere (Doyle et al. 1996a, 1996b). **(B)** Summary of data obtained from 75 *M. musculus* × *M. castaneus* intercross animals. **Top:** Partial map of Chr 8 calculated from intercross data, including those for the segregation of the tg^{la} mutant allele. **Bottom:** Summary of segregation patterns of *tg* and flanking genes. Each column represents the pair of chromosomes inherited by a group of intercross progeny from their *Mus castaneus*/C57BL/6J-tg^{la} × *Mus castaneus*/C57BL/6J-tg^{la} F_1 parents, with black boxes representing C57BL/6J and white boxes representing *M. castaneus* alleles for a given locus. Variant restriction fragments used to follow the segregation of each gene in the 75 intercross progeny were as follows: segregation of *Lpl* was traced with variant *Eco*RV fragments [*M. musculus castaneus* (C) = 10 kb; *M. musculus* C57BL/6J (B) = 12 kb], *Junb* with *Eco*RV[(C) = 11 kb; (B) = 24 kb], *Phkb* with *Msp*I[(C) = 3 kb; (B) = 2 kb], and *Es22* with *Kpn*I [(C) = 2.7 kb; (B) = 3.3 kb]. Distances and standard errors were calculated and are displayed as described in **(A).**

clone isolated (7.75 kb in length) showed 99% nucleotide identity with the rat rbA gene sequence beginning 90 bp from the translational start site, and 90% identity with overlapping portions of an unpublished human calcium channel cDNA fragment, pR5-6cort (Genbank accession no. A22946; data not shown). The intact mouse cDNA probe was hybridized to Northern blots containing polyA+ RNA from (i) normal whole mouse embryos at 10.5, 12.5, 14.5, and 16.5 days-post-coitum (dpc); (ii) whole brains isolated from normal newborn mice; and (iii) separated cerebellum and "rest of brain" from mice at 8, 14, and 18 days of age as well as from 6-month-old animals. We also compared the expression of the *Cacnl1a4* gene in normal mice with age-matched tg^{la} mutants and adult *tg* homozygotes (Fig. 2).

Cacnl1a4 transcripts were detected in mouse embryos as early as 10.5 dpc, with a peak of embryonic expression appearing at 14.5 dpc (Fig. 2A). In addition to a large mRNA species of approximately 8.8 kb, a shorter transcript, approximately 4.5 kb in length, was also detected in embryos at all ages examined (Fig. 2A). Although present data do not permit us to assess the structure of this shorter transcript, an mRNA species of similar size has also been reported to be produced from a closely related calcium channel gene in skeletal muscle of newborn rabbits. The shorter transcript, which is not observed in adult rabbit tissues, has been predicted to encode a protein containing only two of the four membrane-spanning domains present in the adult form of the channel subunit (Malouf et al. 1992).

Once expression is initiated, *Cacnl1a4* continues to be transcribed at high levels in the brains of normal mice into adulthood (Fig. 2B). In contrast to published studies with rabbit (Mori et al. 1991) and rat (Starr et al. 1991), our data indicate that *Cacnl1a4* mRNA is not found in strikingly higher quantities in mouse cerebellum relative to remaining pooled portions of the brain (Fig. 2B, 2C). Longer gel separations showed the ca. 8.8-kb *Cacnl1a4* mRNA to be comprised of two distinct transcripts, approximately 8.5 kb and 9.0 kb in length, respectively (Fig. 2B, 2D). Transcripts of similar size have also been observed in other species (Margolis et al. 1995; Mori et al. 1991; Starr et al. 1991). The two transcripts were present in approximately equal quantities in cerebellum, while the 8.5-kb transcript appeared to be expressed at slightly lower levels relative to the larger species in the "rest of the brain" (Fig. 2B). These observations agree with earlier studies indicating differential expression of the two major α_{1A} calcium channel gene transcripts in various regions of the rat brain (Starr et al. 1991).

Expression in tottering and leaner mutant animals. Cacnl1a4 is abnormally expressed in tg^{la} mutant mice, as demonstrated by data reported below. *Cacnl1a4* mRNA was seen to be present in significantly reduced quantities in brains of tg^{la}/tg^{la} mice (Fig. 2C); longer gel separations showed that the smaller of the two major transcripts was especially reduced in quantity in the mutant

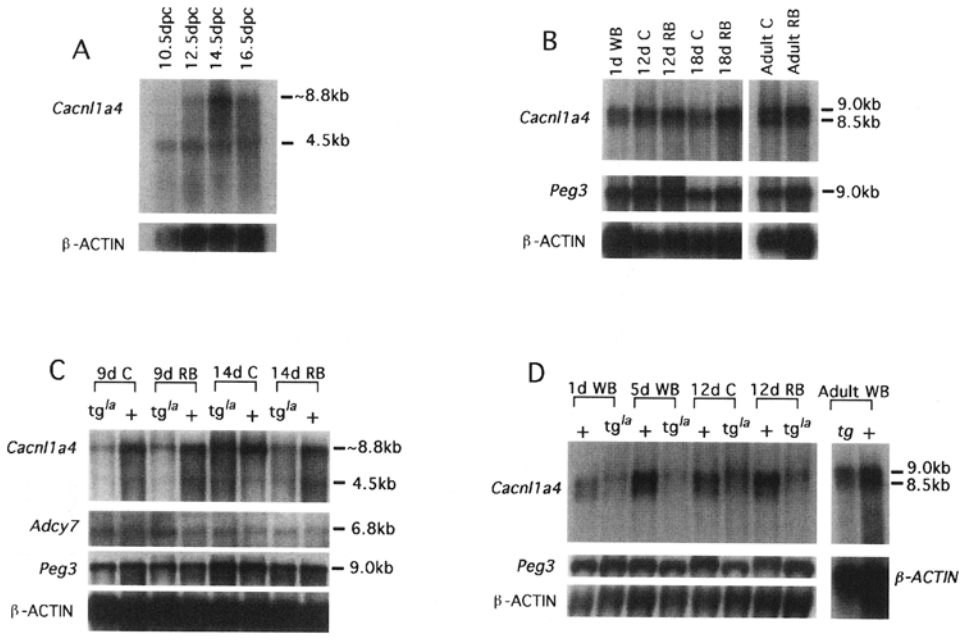
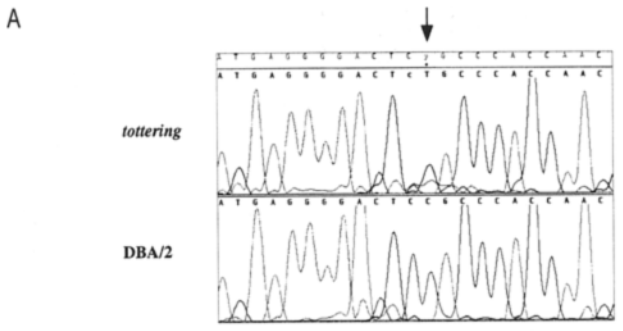


Fig. 2. Expression profiles of *Cacnl1a4* during the course of development in wild type, leaner and tottering mutant mice. All Northerns were hybridized with human β-ACTIN probe to estimate relative quantities of RNA in each lane, as shown in panels below each figure. Estimated sizes of each transcript are presented next to each figure. **(A)** Expression of *Cacnl1a4* in prenatal development. PolyA+ RNA isolated from whole mouse embryos at 10.5, 12.5, 14.5, and 16.5 dpc was hybridized with the *Cacnl1a4* cDNA probe, as described in Materials and methods. Traces of the ca. 8.8-kb transcript were also seen in 10.5 dpc embryos upon longer exposures (data not shown). **(B)** Expression of *Cacnl1a4* in normal newborn, juvenile, and adult mice. PolyA+ RNA prepared from whole brains (WB) of 1-day-old mice, or from cerebellum (C) separated from rest of brain (RB) of 12-day-old, 18-day-old, and 6-month-old animals. *Cacnl1a4* transcripts appear as a doublet of 9.0- and 8.5-kb mRNA species in these longer gel separations. Hybridization patterns of the mouse *Peg3* gene (Kuniowa et al. 1996), which expresses a 9-kb transcript at high levels throughout the murine brain (J. Kim and L. Stubbs, manuscript in preparation), and human β-actin cDNA probes are included below the figure as indicators of the amounts and conditions of RNA samples in each lane. **(C)** *Cacnl1a4* expression in 9- and 14-day leaner (tg^{la}), and normal (+) mice. C = cerebellum, **RB** = rest of brain. Mouse *Adcy7* and *Peg3*, and human β-ACTIN probes were hybridized to the same blot for comparison; hybridization patterns obtained with those probes are shown in panels below the figure. Estimated sizes of transcripts are indicated at the right of the figure. **(D)** Expression of the two major *Cacnl1a4* transcripts in normal and leaner mutant mouse brains. PolyA+ RNA was prepared from whole brains (WB) of normal and leaner (tg^{la}/tg^{la}) mice at 1 day and 5 days after birth, from cerebellum (C) and "rest of brain" (RB) of 12-day-old animals, and from whole brains (WB) of 6-month-old tottering (*tg/tg*) and normal mice. The gel was run long enough to separate the 9.0-kb and 8.5-kb major *Cacnl1a4* transcripts. Hybridization patterns of *Peg3* and β-actin controls are shown in panels below. Estimated sizes of transcripts are indicated at the right of the figure.

animals (Fig. 2D). By contrast, both major transcripts were present at normal levels in the brains of *tg* homozygotes (Fig. 2D). Although tg^{la} mutants display initial signs of granule cell structural abnormalities as early as 8 days after birth, no obvious structural alterations are seen in the cerebellum of newborn animals or in other regions of the tg^{la} brain (Meier and MacPike 1971; Herrup and Wilczynski 1995). Since reduced levels of *Cacnl1a4* transcription were observed in newborn tg^{la}/tg^{la} mice and throughout the brain in mutant animals at all ages examined (Fig. 2C, 2D), we conclude that the reduced levels of expression cannot represent a secondary consequence of the cerebellar degeneration seen in older leaner mice. To provide additional support for this notion, we examined the expression of a closely linked gene known to be expressed in affected cell types. Adenylyl cyclase-7 (*Adcy7*), which is expressed in cerebellar granule cells (Hellevuo et al. 1995), was expressed normally in mutant animals as late as 18 postnatal days (Fig. 2C). These experiments indicate that abnormal *Cacnl1a4* gene expression precedes the massive loss of granule cells, the first sign of cerebellar degeneration observed in leaner mutants (Meier and MacPike 1971; Herrup and Wilczynski 1995).

Sequence analysis of Cacnl1a4 gene regions in normal and mutant mice. Calcium channels contain four closely related domains, each comprised of six membrane-spanning regions, termed S1 through S6. The four domains are thought to interact within the membrane to form calcium-sensing and pore regions of the channel molecule (reviewed by Catterall 1995). Mutations in the conserved membrane-spanning domains are frequently associated with inherited ion channel disorders, including several producing phenotypes that are similar to those expressed by tottering mutants (Ptacek et al. 1991; Browne et al. 1994; Patil et al. 1995). Because the sizes and relative levels of *Cacnl1a4* transcript appeared normal in tottering mutants, we focused upon analysis of the conserved loop domains in our search of point mutation or similar abnormalities in *tg* mutant mice. To examine the structure of these regions, we sequenced RT-PCR products corresponding to each of the four domains from tottering mutant brain RNA samples. The sequence of each mutant domain was then compared with the structure of corresponding regions in RT-PCR products prepared from DBA/2 mice, which represent the inbred background upon which the tottering mutant originally arose.

These experiments revealed the presence of a single cytosine-to-thymidine substitution in tottering mutant animals, which would result in replacement of leucine for a proline residue in the S5-S6 region of conserved domain II in the mutant protein (Fig. 3B). The proline residue that has been replaced in tottering mutants is highly conserved, being present in an analogous position in calcium channel alpha subunits from rat, rabbit, humans, *Caenorhabditis elegans*, the electric ray (*Discopyge ommata*), and the housefly (*Musca domestica;* Fig. 3B). Membrane-spanning domains of calcium channel proteins are generally very well conserved between subtypes and in different species (Catterall 1995). However, although the proline that is altered in tottering mice is conserved in most known calcium channel alpha subunits, amino acid residues immediately neighboring this conserved proline residue are vari-



B

	S5		S6	
Cacnl1a4-*tg*	LFGGQFNFDEGT	LPT	NFDTFPAAIMTVFQILTGEDWNEVMYDGIKSQGGVQGGMVFS	
Cacnl1a4-DBA/2	LFGGQFNFDEGT	PPT	NFDTFPAAIMTVFQILTGEDWNEVMYDGIKSQGGVQGGMVFS	
Human CACNL1A4	LFGGQFNFDEGT	PPT	NFDTFPAAIMTVFQILTGEDWNEVMYDGIKSQGGVQGGMVFS	(X99897)
Rat rbA	LFGGQFNFDEGT	PPT	NFDTFPAAIMTVFQILTGEDWNEVMYDEIKSQGGVQGGMVFS	(M64373)
Rabbit BI-1	LFGGQFNFDEGT	PPT	NFDTFPAAIMTVFQILTGEDWNEVMYDEIKSQGGVQGGMVFS	(X57476)
Electric ray	LFGGQFNFEEGT	PPT	NFDTFPAAIITVFQILTGEDWNEVMYNGIKSQGGVNSGMWSS	(G476977)
C. elegans unc-2	LFGGRFNFPTMH	PYT	NFDTFPVALITVFQILTGEDWNEVMYLAIESQGGIYSGGWPY	(U25119)
Mouse N-type	LFGGQFNFQDET	PTT	IFDTFPAAILTVFQILTGEDWNAVMYHGIESQGGVSKG FS	(U04999)
Rat rb-I	LFGGQFNFQDET	PTT	NFDTFPAAILTVFQILTGEDWNAVMYHGIESQGGVSKGM.FS	(M92905)
Human N-type	LFGGQFNFQDET	PTT	NFDTFPAAILTVFQILTGEDWNAVMYHGIE.QGG.SKGM.FS	(M94173)
Rabbit BIII	LFGGQFNFKDET	PTT	NFDTFPAAILTVFQILTGEDWNAVMYHGIESQGGVSRGM.FS	(D14157)
Mouse alpha-1E	LFGGRFNFNDGT	PSA	NFDTFPAAIMTVFQILTGEDWNEVMYNGIRSQGGVSSGMWSA	(L29346)
Rat rbE-II	LFGGRFNFNDGT	PSA	NFDTFPAAIMTVFQILTGEDWNEVMYNGIRSQGGVSSGMWSA	(L15453)
Human alpha-1E-1	LFGGRFNFNDGT	PSA	NFDTFPAAIMTVFQILTGEDWNEVMYNGIRSQGGVSSGMWSA	(L29384)
Rabbit BII-2	LFGGRFNFNDGT	PSA	NFDTFPAAIMTVFQILTGEDWNEVMYNGIRSQGGVSSGMWSA	(X67856)
Rat alpha-1	LFGGKFNFDETQT	KRS	TFDNFPQALLTVFQILTGEDWNAVMYDGIMAYGGPSSSGMIV	(M57682)
Human alpha-1D	LFGGKFNFDETQT	KRS	TFDNFPQALLTVFQILTGEDWNAVMYDGIMAYGGPSSSGMIV	(M76558)
House fly alpha-1	VFGGRFTFKPEEEK	PRS	NFDSFYQSLLTVFQILTGEDWNVVMYDGIRAYGGVFSFGIVA	(Z31723)

Fig. 3. Sequence analysis of *Cacnl1a4* gene regions in normal and tottering locus mutant mice. **(A)** Electropherogram and resulting sequence of the S5-S6 region of membrane-spanning domain II from tottering mutant (*tg/tg*) and DBA/2 mice. Sequence analysis of the conserved membrane-spanning domain II from tottering mutants revealed a single-base mutation involving the replacement of a C with a T in mutant mice. These results were confirmed by analyzing several additional samples by both automated and manual sequencing methods. **(B)** Predicted amino acid sequence of the domain II S5-S6 region in tottering and DBA/2 mice, compared with sequence from related regions of calcium channel alpha subunit subtypes from a variety of species. The single base pair change distinguishing tottering from DBA/2 mice can be predicted to result in substitution of a leucine for a proline in the S5-S6 interloop region, which is thought to regulate ion conductance and specificity of the channel protein (Catterall 1995). The proline residue affected by the tottering mutant is conserved in $\alpha 1A$, $\alpha 1B$, and $\alpha 1E$ calcium channel alpha subunit genes of rat, rabbit, human, electric ray (*Discopyge ommata*), *Caenorhabditis elegans* (*Unc*-2 gene) and the housefly (*Musca domestica*), suggesting that it plays an important role in channel function. Interestingly, $\alpha 1D$ subunits, which form the core of L-type channel molecules, comprise the only sequenced subtype that does not contain a proline at this otherwise conserved site.

able between species and channel subtypes (Fig. 3B). It is interesting to note that $\alpha 1A$, $\alpha 1B$, and $\alpha 1E$ subunits—found in N-, Q-, and P-type channels, which produce calcium currents of intermediate duration—retain the proline residue at this S5-S6 site. By contrast, $\alpha 1D$ subunit molecules—which form the core of L-type calcium channels, so named because they produce long-lasting calcium currents—are the only known alpha subunit proteins that do not contain a proline at this site.

Human gene mapping. To determine the location of CACNL1A4 in human Chr 19p, we hybridized the 7.75-kb mouse cDNA clone to filter arrays of Chr 19-specific human cosmids under conditions of reduced stringency. These hybridizations identified a series of positive cosmid clones located in three overlapping 19p13.1 contigs that had been previously localized between RFX1 and LYL1 in the distal portion of 19p13.1 (Ashworth et al. 1995; Fig. 4A). To estimate a minimal length for the human CACNL1A4 gene and to determine its transcriptional orientation within 19p, we hybridized the mouse cDNA clone to *Eco*RI restriction digests of a set of cosmids spanning the 136-kb contig region. By comparing the pattern of positively hybridizing fragments with the *Eco*RI restriction map of the region, we were able to determine that CACNL1A4 gene sequences are distributed over a genomic interval of at least 77 kb (Fig. 4B). The centromeric-telomeric orientation of the 136-kb interval had also been determined by Ashworth and colleagues (1995) through high-resolution fluorescence in situ hybridization (FISH) mapping of two cosmids, R29174 and F16894, located at opposite ends of the restriction map (Fig. 4B). To determine transcriptional orientation of the gene, we hybridized a 2.5-kb *Eco*RI fragment of the mouse *Cacnl1a4* cDNA clone, derived from the 3′-end of the mouse clone (and containing the 372-bp conserved DNA fragment described above) to the *Eco*RI-digested cosmids. The results indicated that human CACNL1A4 is transcribed in a centromeric-telomeric direction (Fig. 4B). Since the mouse cDNA clone does not contain the extreme 5′-end of the mouse gene, it is likely that portions of the CACNL1A4 transcription unit are located in cosmids extending further in the proximal direction (Fig. 4B).

These data place CACNL1A4 between polymorphic markers D19S221 and D19S226, which are located approximately 800 kb proximal and 700 kb distal of the calcium channel gene, respectively (Fig. 4A). Published reports have placed EA2, associated with exertion- or stress-induced spells of generalized ataxia, nystagmus, and occasionally cerebellar atrophy in human patients, between D19S413 and D19S226, with the highest LOD score associated with D19S221 (Kramer et al. 1994). The MHP gene has also been mapped to this region (Joutel et al. 1993). Our data, together with the published physical map of human Chr 19 (Ashworth et al. 1995), therefore localize EA2, MHP, and the human α_{1A} calcium channel gene to the same physical interval of Chr 19p. These mapping data, together with links that have been established between channel-encoding genes and other types of human periodic ataxias (reviewed by Griggs and Nutt 1995) suggest CACNL1A4 as an excellent candidate for *EA2* and also for MHP (see below).

Human gene expression. To examine expression of CACNL1A4 in the human brain, we hybridized the conserved 2.5-kb *Eco*RI fragment of the mouse cDNA clone to Northern blots carrying RNA from dissected brain regions. The human gene did appear to be expressed at higher levels in cerebellum than in other regions of the brain. CACNL1A4 transcripts were also observed in cerebral cortex, especially frontal lobe, putamen, spinal cord, amygdala, caudate nucleus, and hippocampus (Fig. 5). Interestingly, subtle anatomical abnormalities have been observed in the tottering hip-

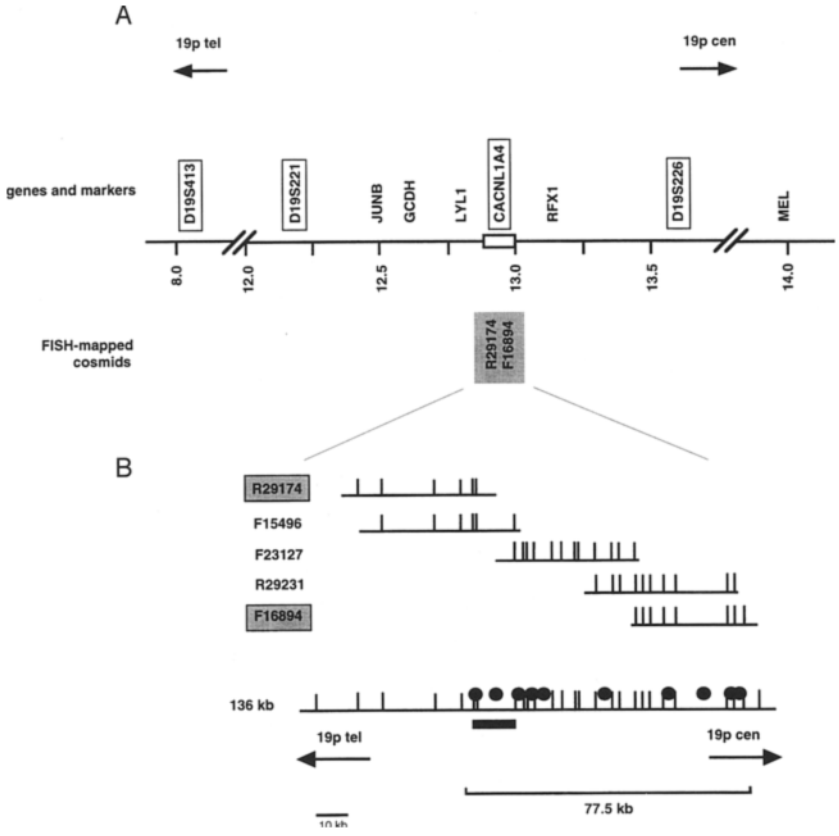


Fig. 4. (**A**) Physical map of the human Chr 19p13.2-13.1 region containing the CACNL1A4 gene. Positions of other genes and markers are taken from previously published reports (Doyle et al. 1996b; Ashworth et al. 1995). The CACNL1A4 gene and three polymorphic markers used to map 19p13 diseases EA2 and MHP are indicated by boxed symbols. Numbers under the map indicate distances from the 19p telomere, in Mb. The positions of FISH-ordered cosmids R29174 and F16894 are indicated in the shaded box under the map. Adjacent pairs of vertical markings define 100-kb intervals. (**B**) *Eco*RI restriction map of CACNL1A4-containing contigs. Cosmids R29174 and F16894 are located at opposite ends of a series of overlapping human clones containing sequences that are recognized by the mouse *Cacnl1a4* cDNA probe. Mouse *Cacnl1a4* cDNA probes were hybridized to *Eco*RI digests of a spanning set of cosmids, and results integrated with the restriction map of the region. Cosmid numbers are shown to the left of the restriction maps for individual, overlapping clones; a summary map incorporating information from all cosmids in this region is given at the bottom of the figure. Dark circles above the summary map indicate the positions of restriction fragments containing sequences that hybridized strongly to the 7.75-kb mouse cDNA probe. The dark bar under the map indicates the positions of two adjacent restriction fragments carrying sequences recognized by a 2.5-kb restriction fragment from the mouse cDNA clone, corresponding to the 3′-end of the gene. These data, combined with the known order of cosmids R29174 and F16894 in 19p13.1, allow us to estimate that CACNL1A4 spans a distance of at least 77 kb and is transcribed in a centromeric-telomeric orientation. Sequences derived from the extreme 5′-end of the gene, which are not present in the mouse cDNA clone, may be located in cosmids extending beyond this 136-kb interval in the centromeric direction.

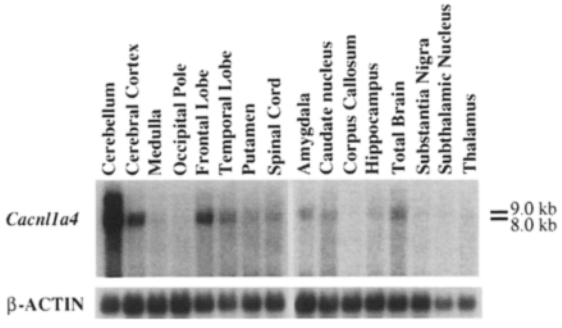


Fig. 5. Expression of CACNL1A4 in specific regions of the human brain. A Northern blot containing polyA+ RNA isolated from dissected human brain regions (Clonetech) was hybridized with the mouse *Cacnl1a4* cDNA probe. A major, broad band, representing mRNA approximately 8.8 kb in length, was detected in several brain regions. The hybridization pattern produced on the same blot by the human β-ACTIN cDNA control is shown in the panel below.

pocampus (Stanfield 1989), and while cerebellum, putamen, and spinal cord are centrally involved in motor control and coordination, abnormal interactions between cortical and thalamic neurons have been suggested as a possible mechanism for focal motor seizures in *tg/tg* animals (Kostopoulos 1992). The pattern of human CACNL1A4 expression is therefore consistent with that expected for a gene associated with mutations causing ataxia and/or seizures in humans and mice.

Discussion

In the course of this study, we have mapped the mouse *Cacnl1a4* gene to the tottering region of central Chr 8 and have localized the human homolog between D19S226 and D19S221 in human Chr 19p13.1. The gene is expressed as early as 10.5 days of mouse embryonic development and continues to be transcribed at high levels into adulthood in normal animals. By contrast, *Cacnl1a4* gene transcripts are present in anomalously low levels in brains of leaner mutants of all ages. Although both major alternative *Cacnl1a4* transcripts are expressed at lower levels in leaner animals, the smaller of the two mRNA species is especially reduced in quantity in the mutant mice. A likely explanation for the selective reduction of the 8.5-kb *Cacnl1a4* transcript is that the leaner mutation disrupts alternative splicing of the gene. Several different alternative versions of α_{1A} calcium channel gene transcripts have been described in previously published reports, including a major splicing event involving insertion or deletion of a 423-bp DNA segment located directly adjacent to a CAC/CCA histidine-encoding repeat region near the 3′-end of the gene. The insert introduces in-frame stop codons that lead to the production of the shorter of two functional α_{1A} subunit isoforms (Mori et al. 1991). Precisely which type of splicing event differentiates the 8.5- and 9-kb major mouse brain *Cacnl1a4* transcripts cannot be determined with present data, but a 423-bp insertion/deletion would be consistent with the estimated lengths of the two mRNA species. Further work defining the molecular basis of the defect observed in leaner mutants will provide important new insights regarding the structures and functions of each of the two major alternative transcripts of the *Cacnl1a4* gene.

The striking differences observed between the *tg* and tg^{la} mutant phenotypes provide the first clues to the distinct functional roles performed by the two alternative *Cacnl1a4* isoforms. Previously published reports have shown that the two major α_{1A} calcium channel gene transcripts are differentially expressed in the rat brain, with the smaller of the two mRNA species especially prominent in rat cerebellum, spinal cord, pons/medulla, hypothalamus/thalamus, and olfactory bulb, but expressed at relatively low levels in striatum, hippocampus, and cortex (Starr et al. 1991). Given these data, it is tempting to speculate that absence of the longer channel isoform, encoded by the 8.5-kb transcript, in CNS regions involved in motor control is responsible for the severe cerebellar ataxia seen in leaner mutants. By contrast, the presence of both channel isoforms throughout the brain, in normal quantities but with altered channel properties, produces a less severe form of ataxia but also is responsible for the focal motor seizures specifically associated with the tottering mutant allele.

The coincident positions of human CACNL1A4, EA2, and MHP may provide additional clues to the varied biological functions of the α_{1A} calcium channel gene. Links that have been established between other types of periodic ataxias and ion channel genes have led to the prediction that EA2 will also be found to be due to an ion channel defect (Vahedi 1995). Similarities between closely linked EA2 and MHP—for example, the fact that some migraine patients show signs of cerebellar atrophy, ataxia and nystagmus, while many EA2 patients experience migraine headaches (Joutel et al. 1993; von Brederlow et al. 1995; Gancher and Nutt 1986; Zasorin et al. 1983)—has prompted the suggestion that the two diseases represent versions of the same highly variable genetic condition (Kramer et al. 1995). Data presented here, therefore, suggest that tottering and EA2, and perhaps also MHP, may represent mutations in an orthologous pair of mouse and human calcium channel genes. If these predictions are borne out by family studies, they provide a link between a diverse set of clinical symptoms—ranging from focal motor and absence seizures to episodic ataxia, cerebellar degeneration, and migraine—as a spectrum of closely related conditions with a common genetic cause. In line with this notion, it is interesting to note that while the CCA repeat region contained within the CACNL1A4 gene is not hypervariable in the human population (Margolis et al. 1995), inheritance of EA2 has shown signs of anticipation in at least one family (Teh et al. 1995); examination of the CACNL1A4 repeat-region structure in this family may, therefore, prove to be especially enlightening. The possible relationship between the CACNL1A4 gene and these 19p-linked diseases, and discovery of the pathways through which specific *Cacnl1a4* gene mutations produce seizures, cerebellar degeneration, and ataxia in mice, represent the first steps toward a better understanding of the genetic and molecular basis of this complex and medically important set of neurological disorders.

Note added in proof: Since completion of this work, concrete proof of CACNL1A4 mutations in both EA2 and MHP families has been documented in a paper published by R.A. Ophoff and colleagues (Cell 87:543-552, 1996). These data confirm our predictions regarding the potential of tottering locus mutations to serve as animal models for both human diseases.

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