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Mutation of the Ca^{2+} Channel β Subunit Gene *Cchb4* Is Associated with Ataxia and Seizures in the Lethargic (*lh*) Mouse

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Summary

Ca^{2+} channel β subunits regulate voltage-dependent calcium currents through direct interaction with α_1 subunits. The β- and α_1-binding motifs are conserved, and all β subunits can stimulate current amplitude, voltage dependence, and kinetics when coexpressed with various α_1 subunits. We used a positional candidate approach to determine that the ataxia and seizures in the lethargic (*lh*) mouse arise from mutation of the β-subunit gene *Cchb4* on mouse chromosome 2. A four-nucleotide insertion into a splice donor site results in exon skipping, translational frameshift, and protein truncation with loss of the α_1-binding site. The lethargic phenotype is the first example of a mammalian neurological disease caused by an inherited defect in a non-pore-forming subunit of a voltage-gated ion channel.

Introduction

Voltage-dependent Ca^{2+} channels in neurons are involved in the regulation of specialized cellular functions such as membrane excitability, rhythmic firing, neurotransmitter release, and plasticity (Tsien and Tsien, 1990; Catterall, 1991; Ghosh and Greenberg, 1995). On the basis of electrophysiological and pharmacological properties, mammalian neuronal Ca^{2+} channels have been classified as L, N, P/Q, R, and T subtypes (Tsien and Tsien, 1990; Ellinor et al., 1993; Sather et al., 1993; Zhang et al., 1993). The subunit composition of these channels remains largely undetermined; however, the ω-conotoxin–sensitive N-type Ca^{2+} channel consists of at least three subunits, α1, $\alpha_2\delta$, and β, that are sufficient to reconstitute many of the pharmacological and functional properties of native N-type channels when coexpressed in vitro (Witcher et al., 1993). The α_1 subunit is a transmembrane protein that is sufficient for channel permeability, gating, and voltage dependence (Mikami et al., 1989; Perez-Reyes et al., 1989). Six genes have been identified that encode α_1 subunits (Snutch et al., 1990; Ellinor et al., 1993; Soong et al., 1993; Zhang et al., 1993). Mutations in the α_{1A} gene have recently been associated with inherited neurological disease, including ataxia and seizures in the mouse mutant tottering (*tg*) (Fletcher et al., 1996), and familial hemiplegic migraine (FHM) and episodic ataxia type-2 (EA-2) in human pedigrees (Ophoff et al., 1996).

The importance of the remaining subunits in membrane excitability and synaptic signaling in neurons has not yet been established. Calcium channel β subunits are cytoplasmic proteins that are capable of modulating current amplitude, activation and inactivation kinetics, and voltage dependence when coexpressed with α_1 subunits in Xenopus oocytes or mammalian cells (Lacerda et al., 1991; Singer et al., 1991; Castellano et al., 1993; Ellinor et al., 1993; Stea et al., 1993; Wiser et al., 1996). The site of interaction between α_1 and β subunits consists of an 18 amino acid motif with 9 conserved residues (α_1) and a highly conserved 30 amino acid motif (β) (Pragnell et al., 1994; De Waard et al., 1994, 1995). Four genes have been identified that encode β subunits (Castellano and Perez-Reyes, 1994). All four of these β subunits are able to modulate the functional properties of various α_1 subunits in oocyte coexpression studies, yet it is unclear which combinations occur in vivo (Snutch et al., 1990; Tomlinson et al., 1993; Zhang et al., 1993, De Waard et al., 1994). In situ hybridization studies of brain demonstrate complex spatial and temporal expression patterns for each of the six α_1- and four β-subunit genes, indicating that many different α_1-β combinations may be possible (Williams et al., 1994; Tanaka et al., 1995).

Recently, the gene encoding the voltage-gated Ca^{2+} channel β_4 subunit (*Cchb4*) was localized to proximal mouse Chromosome 2 by analysis of the progeny of two interspecific mouse backcrosses (Chin et al., 1995). This region also contains the neurological mutation lethargic (*lh*), which was mapped approximately 28 cM from the centromere (Southard, 1973; Mouse Genome Database 3.1). Lethargic arose spontaneously in the mouse inbred strain BALB/cGn in 1962. Homozygotes are recognizable at 15 days of age by ataxia and lethargic behavior followed within a few days by the onset of spontaneous focal motor seizures (Dickie, 1964; Sidman et al., 1965). A second seizure type, consisting of brief episodes of behavioral immobility, is accompanied by generalized cortical spike-wave discharges. Electrophysiologically and pharmacologically, these seizures resemble the absence seizures of human petit mal epilepsy (Noebels and Sidman, 1982; Hosford et al., 1992) and those seen in the tottering mouse (Noebels and Sidman, 1979). No pathological changes have been observed in the brain, spinal cord, or muscles of affected lethargic mice (Dung and Swigart, 1972). The interval from 15 days to 2 months of age appears to define a critical period during which, in addition to ataxia and seizures, *lh/lh* mice experience a reduction in body weight relative to unaffected littermates, immunological problems including splenic and thymic involution and severe lymphocytopenia, and increased mortality. Affected mice that survive past two months of age recover much of their immune function and body weight and live normal lifespans, but exhibit reduced fertility (Dung and Swigart, 1971, 1972; Dung, 1977).

The proximity of mouse *Cchb4* to the *lh* locus, its

expression in brain (Castellano et al., 1993), the demonstrated function of Ca^{2+} channel β subunits as regulators of electrical excitability, and the similarity of the lethargic and tottering phenotypes suggested that *Cchb4* was an excellent candidate gene for the *lh* locus. Here, we report that the expression of *Cchb4* is abnormal in *lh/lh* mice and show that this is due to a mutation within the *Cchb4* gene that is consistent with a complete loss of protein function. These results demonstrate the physiological significance of the Ca^{2+} channel β_4 subunit in neurons and establish the importance of ion channel modulatory subunits in mammalian neurological disease.

Results

Cchb4 mRNA Is Reduced in *lh/lh* Brain

We designed oligonucleotide primers based on the published cDNA sequence of rat *Cchb4* (Castellano et al., 1993) and used them to amplify fragments of the mouse *Cchb4* cDNA by RT-PCR of brain RNA. These fragments were sequenced to confirm their identity, radiolabeled, and hybridized to a Northern blot containing polyA$^+$ RNA from +/+ and *lh/lh* brain (Figure 1). A single transcript of approximately 9.4 kb was present in all samples, but at greatly reduced levels in *lh/lh* brain. To identify the cause of this decrease, we examined the *Cchb4* transcript at higher resolution by RT-PCR. The positions of the RT-PCR primers relative to the *Cchb4* cDNA are shown in Figure 2A. Using two sets of primers, the amplification products obtained from lethargic brain RNA were shorter than those from wild-type brain (Figure 2B). The data localize the abnormality in the lethargic transcript to the 244 bp segment between primers pB and pD. *Cchb4* RT-PCR product of wild-type size could not be detected in lethargic brain RNA.

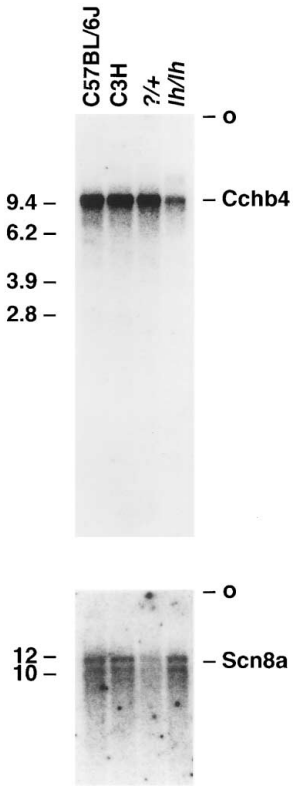

Figure 1. Reduced Abundance of the *Cchb4* mRNA in *lh/lh* Brain

Brain polyA+ RNA (2 μg) from mice of the indicated genotypes was analyzed by Northern blot analysis. The top panel was probed with radiolabeled *Cchb4* RT-PCR products that were amplified from mouse brain RNA. The abundance of the 9.4 kb *Cchb4* transcript was reduced to approximately 20% of normal levels in the *lh/lh* sample. The filter was stripped and rehybridized with a probe for the sodium channel gene *Scn8a* (Burgess et al., 1995) in order to compare the concentration and quality of the RNA samples. The ?/+ control lane contains less RNA than the other lanes. The positions of molecular size standards (kb) are shown at the left. *lh/lh*, lethargic homozygote; ?/+, unaffected littermate; 0, origin.

Tissue Distribution of *Cchb4* mRNA in Wild-Type Mice

Since pathology has been described in nonneuronal tissues of lethargic mice, the tissue distribution of *Cchb4* expression was examined by RT-PCR with primers pB and pD. In addition to brain (Figure 2B), expression of *Cchb4* was detected in wild-type spinal cord, kidney, testis, ovary, and skeletal muscle (Figure 2C). Expression was not detected in heart, liver, lung, spleen, or thymus.

Mutation of a 5′ Splice Site in *lethargic* *Cchb4* Results in Exon Skipping

To determine the basis for the size difference between lethargic and +/+ *Cchb4* brain RT-PCR products, they were cloned and sequenced. Two distinct products, differing in size by 20 bp, were identified in the *lh/lh* sample and designated lh-1 and lh-2. Alignment of the normal and mutant sequences confirmed the partial loss of coding sequences in lh-1 and lh-2 relative to wild type, along with an altered translational reading frame in both *lh* transcripts that introduced termination codons shortly thereafter (Figure 3). The presence of two distinct lethargic RT-PCR products suggested the involvement of aberrant pre-mRNA processing, so we analyzed the splice sites in the corresponding genomic DNA. DNA from mouse strain BALB/cJ was used as the control for genomic analysis since it is closely related to the inbred strain BALB/cGn, in which the lethargic mutation originally arose. The 4 kb region between primers pB and pD was amplified by PCR of *lh/lh* and BALB/cJ genomic DNA, cloned, and partially sequenced. Four exons, designated A, B, C, and D, account for all of the transcribed sequences identified between primers pB and pD in the wild-type *Cchb4* transcript. An insertion of four nucleotides that disrupts the consensus 5′ splice site of intron A/B was identified in the *lh/lh* sequence (Figure 4).

The 4 bp Insertion Is Specific for Lethargic Mice

Primer pJ from exon B and primer pK from the downstream intron were used to detect the lethargic mutation directly by PCR. Amplification of genomic DNA from mice of BALB/cJ and other inbred strains generated the predicted 75 bp product. PCR of genomic DNA from lethargic homozygotes generated a unique 79 bp product, demonstrating the presence of the 4 bp insertion (Figure 5).

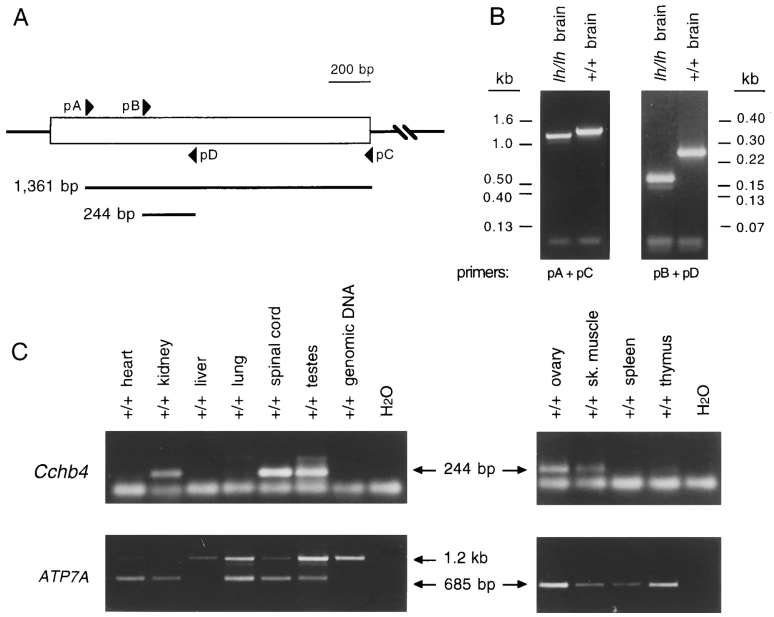

Figure 2. Expression of *Cchb4* in Mutant and Wild-Type Tissues by RT-PCR

(A) The position of the PCR primers relative to the rat *Cchb4* cDNA. PCR amplification of mouse *Cchb4* cDNA with primer pairs pA and pC or pB and pD was predicted to result in products of 1,361 bp or 244 bp, respectively. Open box, coding region; solid line, untranslated regions.

(B) RT-PCR of mutant and wild-type brain RNA. RT-PCR using the indicated primer pairs amplified products from +/+ RNA of the expected sizes. Products amplified from *lh/lh* RNA using the same primers were 50–100 bp smaller.

(C) RT-PCR of RNA from several wild-type tissues using primers pB and pD. Expression was detected in kidney, spinal cord, testis, ovary, and skeletal muscle. Primers that amplify a 685 bp fragment of the mouse *Atp7a* transcript, which is expressed in all cell types except liver (Vulpe et al., 1993), were used as a control. The *Atp7a* primers also amplified a 1.2 kb product that is specific to the presence of genomic DNA as a contaminant in several of the RNA samples. The molecular size standard in (B) and (C) was 1 kb DNA ladder (GIBCO, BRL); +/+, C57BL/6J.

To further characterize the gene structure of mouse *Cchb4*, we used PCR between exons to determine the size of introns A/B, B/C, and C/D. Amplification of either +/+ or *lh/lh* genomic DNA between exons A and B (primers pB and pH) yielded a product of 2.3 kb, between exons B and D (primers pG and pD) a product of 1.7 kb, and between exons A and D (primers pB and pD) a product of 4.0 kb (not shown). The distance from exon C to exon D was determined by partial sequencing of the 4.0 kb genomic PCR product. The deduced partial gene structure of mouse *Cchb4* and a model summarizing the *lh* defect are shown in Figure 6. The mutation leads to translational frameshift and the predicted loss of >60% of the Cchb4 protein C-terminus, including the essential α_1-binding motif located immediately 3′ to the mutation.

Discussion

In this study, we evaluated the mouse Ca^{2+} channel β_4 subunit gene *Cchb4* as a positional candidate for the lethargic locus on proximal mouse Chr 2. The initial observation of reduced abundance of the *Cchb4* transcript in *lh/lh* brain led to the identification of a four-nucleotide insertion within a 5′ splice site in *lh/lh* mice. This insertion was the only sequence difference we observed between *lh/lh* and BALB/cJ in the regions of the *Cchb4* gene that were compared. The 77 bp exon preceeding the mutation is consistently skipped during *Cchb4* pre-mRNA processing in *lh/lh* brain but not in wild-type brain. Similar mutations in the 5′ splice sites of other genes, including those resulting in a "T" at position +3 instead of the consensus "A" or "G", have also been shown to affect pre-mRNA processing (Table 1; Nakai and Sakamoto, 1994). The 20 bp exon downstream of the mutation is also skipped in a fraction of transcripts in *lh/lh* brain. Since the homologous 20 bp exon is alternatively spliced in the evolutionarily conserved rat Ca^{2+} channel β_{1-4} genes and the mouse Ca^{2+} channel β_3 gene (Castellano and Perez-Reyes, 1994; Murakami et al., 1996), skipping of this exon in the β_4 gene of lethargic mice may not be a specific property of the mutant allele. The two aberrant *Cchb4* transcripts we identified in *lh/lh* brain both result in translational frameshifts and are predicted to encode truncated β_4 proteins missing 60% of their C-terminus relative to wild type, including the highly conserved α_1 subunit–binding site (Figure 6C). Since experiments in Xenopus oocytes have shown that β_{1b} subunits without this motif failed to stimulate calcium current when coexpressed with α_{1A} and α_{2b} subunits (De Waard et al., 1994), we conclude that the lethargic locus is likely to represent a functionally null allele of the *Cchb4* gene.

Molecular Pathology of the Lethargic *Cchb4* Mutant Phenotype

In situ hybridization analysis of developing rat brain demonstrates that *Cchb4* is diffusely expressed in the mantle but not ventricular zones of the neuraxis at embryonic stages and is then selectively attenuated during postnatal development. From postnatal day 14 onward, *Cchb4* is expressed predominately in cerebellar Purkinje and granule cells, and at lower levels in olfactory bulb, hippocampus, cerebral cortex, and thalamic nuclei (Tanaka et al., 1995). Ca^{2+} channel signaling defects in these circuits are remarkably congruent with the anatomical substrate of the lethargic neurological phenotype of ataxia, focal motor seizures, and cortical spike-wave epilepsy.

Ataxia is a consistent result of abnormalities in Purkinje and granule cells, and these two cell populations show the strongest levels of *Cchb4* expression in the brain. Loss of β_4 function in the substantia nigra, red nucleus, inferior olivary, and cerebellar deep nuclei

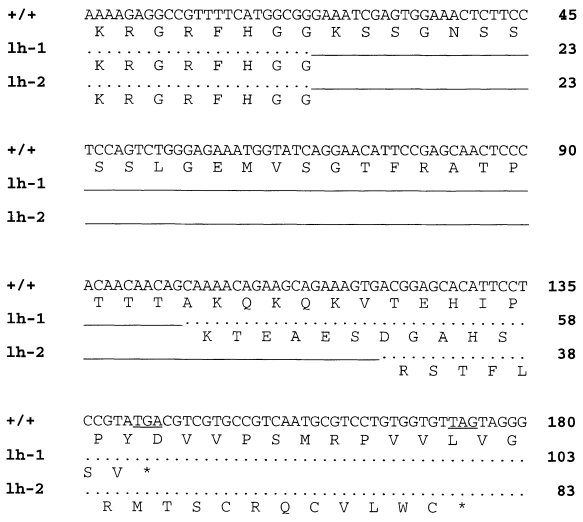

Figure 3. Alignment of Wild-Type and Mutant *Cchb4* Partial cDNA Sequences

RT-PCR products amplified with primers pB and pD (Figure 2A) from total brain RNA of wild-type (+/+) and *lh/lh* mice were cloned and sequenced, and the sequences were aligned. The conceptual translations are indicated below the nucleotide sequences. The 180 bp +/+ mouse product corresponds to nucleotides 698–877 of the rat *Cchb4* cDNA sequence (GenBank accession number L02315). Two different classes of RT-PCR products were isolated from *lh/lh* brain RNA. lh-1 and lh-2 are 77 bp and 97 bp smaller than the +/+ product, respectively. The apparent deletion of internal sequences leads to shifts in the translational reading frame relative to +/+ in both transcripts. The termination codon that would be encountered in the mutant transcripts is underlined in the +/+ sequence and indicated by an asterisk in the predicted amino acid sequences. Dots indicate nucleotide identity; +/+, C57BL/6J.

could also contribute to the slowed, ataxic gait. These pathways are likely to contribute to the second element of the lethargic syndrome, intermittent episodes of repetitive limb shaking. The rapid clonic limb movements were initially considered to be focal motor seizures; however, electrocorticographic recordings in *lh/lh* mice during these episodes fail to reveal abnormal cortical discharges (J. L. N., unpublished data), indicating a subcortical origin. Therefore, this episodic behavior may be more accurately termed a focal myoclonus or a paroxysmal dyskinesia. Calcium currents play a critical role in thalamic oscillations and cortical synchrony, and their involvement has been directly implicated in the generation of cortical spike waves. This discharge pattern is specific for nonconvulsive spike-wave absence seizures, a major form of inherited childhood epilepsy that differs physiologically and pharmacologically from convulsive seizure types. The reciprocal connections between pyramidal neurons in deeper layers of the neocortex, cortical relay neurons in the thalamus, and their respective interneurons are believed to form the elementary pacemaking circuit. Current hypotheses regarding the generation of this aberrant oscillation focus on the control of intrinsic rhythmic membrane firing properties by the low voltage–activated or T-type Ca^{2+} current in both relay cells (Jahnsen and Llinas, 1984; Huguenard and Prince, 1994) and the inhibitory neurons of the GABAergic nucleus reticularis thalami (Avanzini et al., 1986; Huguenard and Prince, 1992). Enhancement of this current has been detected in one genetic model of spike-wave epilepsy in the rat (Tsakiridou et al., 1995), and it is reduced by ethosuximide, an antiepileptic drug highly selective for the spike-wave pattern of epilepsy (Coulter et al., 1989). Since *Cchb4* is expressed in wild-type neocortex and thalamus, defective modulation of calcium currents produced by the lethargic mutation could favor abnormal oscillations in these circuits.

Nonneuronal expression of mouse β_4 was detected in wild-type kidney, testis, ovary, and skeletal muscle by RT-PCR. Interestingly, pathological examination of *lh/lh* mice surviving to adulthood reveals evidence of renal cysts (Dung and Swigart, 1971), suggesting that *Cchb4* may play a role in this aspect of the lethargic phenotype. It is noteworthy that the human-inherited polycystic kidney disease PKD2 is caused by mutations in a gene with homology to voltage-dependent Ca^{2+} channel α_1 subunits (Mochizuki et al., 1996). Male and female *lh/lh* mice exhibit reduced fertility, which may be related to loss of β_4 function in the testis and ovary. While no myopathic changes have been reported in lethargic homozygotes, expression of *Cchb4* in skeletal muscle is consistent with a potential defect in voltage-gated calcium channel activity in that tissue. No *Cchb4* expression was detected in wild-type spleen or thymus under our assay conditions, and therefore the relationship between mutation of *Cchb4* and the splenic and thymic involution reported in *lh/lh* mice (Dung and Swigart, 1971, 1972) will require further investigation.

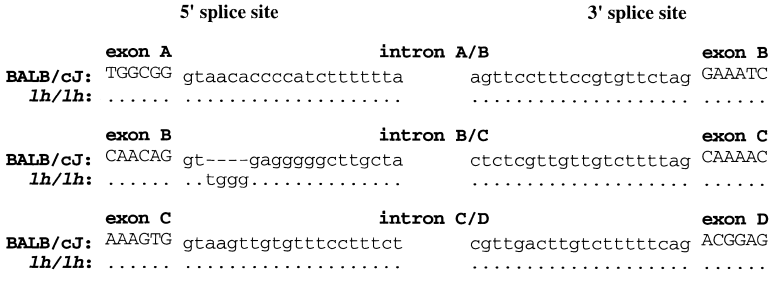

Figure 4. Mutation of a 5′ Splice Site in the *lh/lh Cchb4* Gene

PCR products spanning the genomic region corresponding to the aberrant *lh/lh Cchb4* transcripts were amplified from *lh/lh* and BALB/cJ genomic DNA using primers pB and pD, cloned, and sequenced. Four exons were identified by alignment of the genomic and cDNA sequences and the presence of consensus 5′ and 3′ intronic splice sites at points of discontinuity in the alignment: A, B, C, and D (5′–3′). The three introns were designated intron A/B, intron B/C, and intron C/D. The aberrant *lh/lh* RT-PCR products (Figure 3) correspond to an absence of the sequences contained in exon B (lh-1) and exon B plus exon C (lh-2). The only difference detected between the *lh/lh* and BALB/c genomic sequences was an insertion of four bases, tggg, at position +3 of the consensus 5′ splice site of intron B/C in the *lh/lh* genomic DNA. Superscript capital letters, exon sequences; lowercase letters, intron sequences; dots, nucleotide sequence identity; dashes, gaps inserted to maintain optimal sequence alignment.

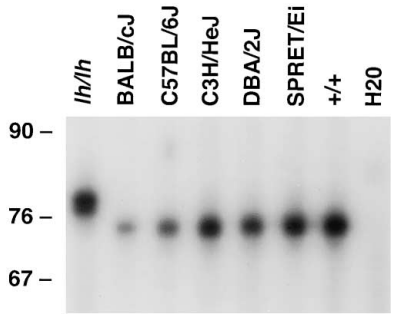

Figure 5. Genotyping the Lethargic Mutation by PCR

Genomic DNA from the indicated strains was amplified in the presence of one radiolabeled primer, and products were fractionated on a 6% polyacrylamide/urea gel. The gel was exposed to X-ray film for 1 hr. *lh/lh*, lethargic homozygote; +/+, unaffected littermate; molecular size marker, pBR322 digested with HpaII.

Functional Diversity of Potential Calcium Current Defects in β Channelopathies

The neurological syndrome of the *lh* mutant demonstrates an essential role of the β_4 subunit in voltage-gated Ca^{2+} channel function and predicts an unusually diverse set of cellular excitability phenotypes in central neuronal pathways. The complexity arises from the range of α_1 subunits expressed in most neurons and the potential promiscuity each may exhibit with the remaining wild-type β subunits coexpressed in the cell. Unlike conventional mutations in single α_1 ion channel subunits that create a uniform defect in a specific calcium current, the loss of a β subunit may lead to altered calcium currents in two general ways. In the first model, loss of a particular β subunit could give rise to a simple channelopathy consisting of a major reduction in calcium current in each α_1 subunit with which it normally interacts. These alterations are predictable based on coexpression studies in oocytes and would generally reduce the magnitude of the peak calcium currents in any of the five neuronal α_1 subunits from 3- to 20-fold, and produce β subunit–specific shifts in the kinetics and voltage dependency of activation and inactivation (Castellano et al., 1993; De Waard et al., 1995).

Alternatively, mutation of a single β subunit could lead to a more complex (or multiplex) channelopathy where, for example, loss of the high affinity β_4 allows a lower affinity β_{1-3}, wherever present, to replace it. These surrogate β-subunit interactions could modify calcium channel kinetics and neuronal signaling in novel and relatively unpredictable ways throughout the brain. For example, while all four β subunits can modulate activation kinetics similarly, there is variation in the modulation of inactivation kinetics (Castellano and Perez-Reyes, 1994; Olcese et al., 1994). It is therefore possible that loss of β_4 and replacement by other β subunits could allow affected channels to express more "T-like" currents, thus favoring rebound bursting and oscillations in thalamic circuits. Since β_4 contains an ATP/GTP-binding site consensus sequence at amino acids 168–176 that is not found in the other β subunits (Castellano et al., 1993), the binding of alternative β subunits to the α_1 subunit might replace the Ca^{2+} current stimulation by β_4, but not its ability to be modulated by intracellular signals.

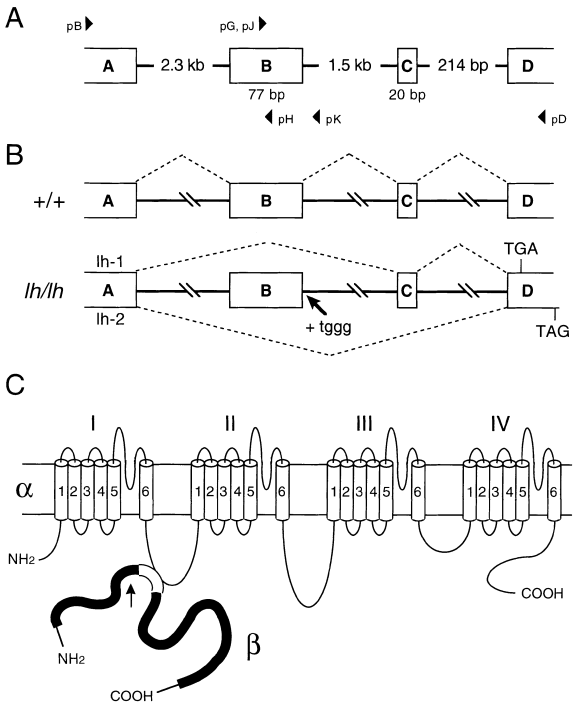

Figure 6. Model of the Defect in the Lethargic *Cchb4* Gene

(A) The partial gene structure of mouse *Cchb4* determined by PCR of genomic DNA and alignment of genomic and RT-PCR product sequences. The size of intron A/B (2.3 kb) was determined by genomic PCR with primers pB and pH. The size of intron C/D was determined directly from sequencing a 4.0 kb genomic PCR product generated with primers pB and pD. The size of intron B/C was inferred from the size of the PCR product of primers pD and pG (1.7 kb) minus the size of intron C/D (214 bp). PCR with primers pJ and pK detects the presence of the 4 bp insertion in lethargic genomic DNA (Figure 5). Primers pG and pJ differ by 1 bp.

(B) The dashed lines indicate the splicing observed in wild-type (+/+) and *lh/lh* brain *Cchb4* transcripts. The location of the four-base-pair insertion in the 5′ splice site of the lethargic *Cchb4* gene is indicated by an arrow. The positions of the termination codons in the frameshifted lh-1 and lh-2 transcripts are indicated in exon D of the *lh/lh* gene structure.

(C) A schematic representation of the interaction of a Ca^{2+} channel α_1 subunit and β subunit (based on De Waard et al., 1995). The binding site for the α_1 I-II cytoplasmic loop is indicated by the white segment of the β subunit and is coded for beginning in exon D of the partial *Cchb4* gene structure. The arrow indicates the relative position of the two translational frameshifts in the predicted lethargic Cchb4 proteins, both of which are predicted to result in a severely truncated protein missing the α_1-binding site and loss of Cchb4 regulatory function.

The above models are not mutually exclusive, and both may contribute to the molecular phenotype. The second model depends on the likelihood of monogamous, or at least preferred, associations between specific α_1 and β subunits. While the native subunit compositions of most Ca^{2+} channels are unknown, three lines of evidence suggest that preferential β–α_1 interactions do exist, at least for β_4. Expression analysis indicates a close fit between the patterns of maximal brain mRNA expression of the α_{1A} and β_4 subunit genes (Tanaka et al, 1995), and this spatial pairing would be entirely consistent with affinity data obtained from in vitro–binding

Table 1. Consensus and Mutant 5′ Splice Sites Compared to the Lethargic 5′ Splice Site Mutation

Gene	Strain/Mutation	5′ Splice Site	Result	Reference
Consensus	+/+	(C/A)AG gt(a/g)agt		Stephens and Schneider, 1992
Mouse *Cchb4*	BALB/cJ	CAACAG gt----gaggggggc		
	lh	.....tggg.....	exon skipping	Present study
Mouse *Scn8a*	+/+	TGTGGA gtaagtaacacttc		
	med^{J}	.....	exon skipping	Kohrman et al., 1996
Human *APRT*	normal	CTGGTG gt-aagggtc		
	APRT deficiency	.....t.....	exon skipping	Hidaka et al., 1987
Human *CHM*	normal	AGATAG gt-aagaaaa		
	choroideremia	.....t.....	intron retention	Sankila et al., 1992
Mouse *H2-Eb*	*H2-Eb*^{b}, *H2-Eb*^{d}	CCAGAC gt-aagtg		
	H2-Eb^{w17}	.....t.....	mRNA absent	Vu et al., 1988

assays of radiolabeled β subunits to α_1 subunit fusion proteins. Scatchard analyses provide a rank order of binding affinity of each β subunit to the α_1 site that is $\beta_4 > \beta_{2a} > \beta_{1b} >> \beta_3$ (De Waard et al., 1995). The β_3 affinity is at least one order of magnitude lower than β_4. Finally, the neurological phenotype of the lethargic mouse is essentially a mirror image to that of the mutant mouse tottering, recently reported to bear mutations in the α_{1A} (P/Q-type) calcium channel gene (Fletcher et al., 1996). Despite this circumstantial evidence for β_4-α_{1A} pairing, in many regions, β_4 is coexpressed with other α_1 subunits, and thus its absence could also reduce the corresponding N-, L-, or T-type Ca^{2+} currents.

The ion-conducting subunits of other important transmembrane cationic channels also coassemble with modulatory subunits and are involved in hereditary neurological disease. For example, the voltage-gated sodium channel α-subunit gene *Scn8a* is mutated in mouse motor endplate disease (*med*) (Burgess et al., 1995), and the voltage-gated potassium channel α-subunit gene *KCN1A* is mutated in human episodic ataxia/myokymia (Browne et al., 1994). The lethargic mutant is the first example of a novel class of hereditary channelopathy that can generate a spectrum of disease phenotypes in the mammalian central nervous system based on defective modulatory subunit interactions.

Experimental Procedures

Northern Analysis of *Cchb4* Expression

Total RNA was prepared from brain tissue using Trizol reagents (GIBCO–BRL). PolyA$^+$ RNA was prepared from total RNA using PolyATract reagents (Promega). PolyA$^+$ RNA (2 μg/lane) was electrophoresed through 1.2% agarose in the presence of 6% formaldehyde and transferred to Zetaprobe GT nylon membrane (BioRad). The membrane was hybridized with radiolabeled RT-PCR products of mouse brain *Cchb4* corresponding to nucleotides 414–1774 (primers pA to pC, coding region) and 2506–3484 (primers pE to pF, 3′ untranslated region) of rat *Cchb4* (Castellano et al., 1993; GenBank accession number L02315). The probe was stripped from the membrane with boiling 0.1× SSC, 0.5% SDS, and the membrane was rehybridized with radiolabeled *Scn8a* RT-PCR products corresponding to nucleotides 235–771 and 3391–4488 (Burgess et al., 1995; GenBank accession number U26707). Probes were radiolabeled with [α-^{32}P]-dCTP (Redivue, Amersham) using the Rediprime DNA labeling system (Amersham).

RT-PCR of *Cchb4* from Wild-Type and Lethargic Tissues

RNA isolation and RT-PCR was carried out by two methods: (A) Brain, heart, liver, lung, spinal cord, and testes were homogenized using a Tissue Tearor (Biospec Products) and total RNA was isolated using the RNeasy Mini Kit (Qiagen). The integrity of the RNA was examined by agarose gel electrophoreses and ethidium bromide staining. Approximately 1 μg of RNA was used for reverse transcription reactions containing 8 ng/μl random hexamers, 50 pM primer pF (specific for 3′ UTR of mouse and rat *Cchb4*), 1 mM each dATP, dCTP, dGTP, dTTP, 4 mM sodium pyrophosphate, 1 unit/μl RNasin, 0.6 units/μl AMV-RT, 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM MgCl2, 0.5 mM spermidine, and 10 mM DTT (Promega). (B) Total RNA was isolated from ovary, skeletal muscle (gastrocnemius), spleen, and thymus with guanidine isothiocyanate or with Trizol reagent (GIBCO–BRL). Five μg aliquots of total RNA were converted to first strand cDNA with random primers using MMLV reverse transcriptase (GIBCO–BRL). PCR reactions contained 1 μl RT reaction or 100 ng genomic DNA as template, 10 mM Tris-HCl (pH 8.3) 1.5 mM $MgCl_2$, 50 mM KCl, 0.1 mg/ml gelatin, 0.2 mM each dATP, dCTP, dGTP, dTTP, 0.5 μM each primer, and 1–1.5 units Taq polymerase (Fisher). Reactions were performed with a PTC-100 thermocycler (MJ Research) with the following conditions: 94°C (2 min); 25 or 30 cycles (RNA set B and A, respectively) of 94°C (20 sec), 62°C (35 sec), 72°C (1 min); final extension 72°C (5 min). The primers were from the University of Michigan DNA Synthesis Core Facility (pA, pC) or from Genosys (pB, pD, pE, pF): pA (5′ CGATTCGGCAAG AGAGAGAACAGCAAG 3′), pB (5′ TGGCTTCATCCCAAGTCCACT GCG 3′), pC (5′ GTTTGGGCAGCCTCAAAGCCTATGTCG 3′), pD (5′ CAGTGATGGCCCTACTAACACCAC 3′), pE (5′ AGCCAGTTTGAAGT TGACAAAATCCAG 3′), pF (5′ TTCAGGCAGCCTCATTATCAGATA AGC 3′). Control primers correspond to nucleotides 613–633 and 1297–1277 of the human *ATP7A* gene (Vulpe, et al. 1993; GenBank accession number L06133). PCR products were cloned into the vector pCR 2.1 using the TA Cloning Kit (Invitrogen) and transformed into InvaF' E. coli competent cells (Invitrogen) by heat shock prior to sequencing. Brain RT-PCR products amplified with primers pA and pC were resolved on 1.2% agarose gel (GIBCO–BRL). Brain RT-PCR products amplified with primers pB and pD were resolved on 3.0% MetaPhor agarose gel (FMC BioProducts).

Analysis of Genomic PCR Products

Genomic PCR was carried out using the Expand Long Template PCR System (Boehringer Mannheim). Reactions contained 200 ng genomic DNA, 0.3 μM each primer, 0.36 mM each dATP, dCTP, dGTP, dTTP, 1.75 mM $MgCl_2$, and 2.6 units of Expand DNA polymerase mix. Reactions were carried out as follows: 94°C (2 min); 9 cycles of 94°C (10 s), 63°C (30 s), 68°C (7 min); 20 cycles of 94°C (10 s), 64°C (30 s), 68°C (7 min + 20 s per cycle), final extension 68°C (7 min). Product sizes were determined by electrophoreses through 0.8% agarose gels. Genomic PCR products were cloned and transformed into E. coli as described for RT-PCR products. Primers (Genosys): pG (5′ AAATGGTATCAGGAACATTCCGAG 3′), pH (5′ TTTTGCTGTTGTTGTGGGAGTTGC 3′).

Genotype Assay

Primer pJ (5′ AAATGGTATCAGGAACATTCCGAGC 3′) and primer pK (5′ CAAACCAGTGAAAGCGTTAGCAAGC 3′) were synthesized by the Oligonucleotide Synthesis Core of the University of Michigan. Primer pJ was end-labeled with [γ-^{32}P]ATP (Amersham) using T4 polynucleotide kinase (Boehringer Mannheim). PCR reactions were incubated at 94°C for 2.5 min followed by 31 cycles of 45 s each at

94°C, 55°C and 72°C, using a PTC-100 thermal cycler (MJ Research). Products were detected by autoradiography after electrophoresis on 6% polyacrylamide/urea gel. The molecular weight marker, pBR322 digested with HpaII, was labeled by the Klenow fragment of DNA polymerase (Boehringer Mannheim) in the presence of [α-^{32}P]dCTP (Amersham).

Sequence Analysis

DNA sequencing was carried out by the University of Michigan Sequencing Core (R. Lyons, Director) and at Baylor College of Medicine. Sequence analysis was performed using MacVector 3.5 software (International Biotechnologies, Inc.). The sequence of the BALB/cJ and *lh*/*lh* intron B/C splice site was confirmed by sequencing genomic PCR products from three independent reactions. Sequence was confirmed by sequencing both the strands. These sequences have been submitted to GenBank (accession numbers U80985 and U80986). The C57BL/6J and *lh*/*lh* brain RT-PCR product sequences were each confirmed by sequencing products from at least two independent reactions.

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