Genetic and Developmental Characterization of *Dmca1D*, a Calcium Channel α_1 Subunit Gene in *Drosophila melanogaster*

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ABSTRACT

To begin unraveling the functional significance of calcium channel diversity, we identified mutations in Dmca1D, a Drosophila calcium channel α_1 subunit cDNA that we recently cloned. These mutations constitute the *l(2)35Fa* lethal locus, which we rename *Dmca1D*. A severe allele, *Dmca1D^{X10}*, truncates the channel after the IV-S4 transmembrane domain. These mutants die as late embryos because they lack vigorous hatching movements. In the weaker allele, *Dmca1D^{AR66}*, a cysteine in transmembrane domain I-S1 is changed to tyrosine. *Dmca1D^{AR66}* embryos hatch but pharate adults have difficulty eclosing. Those that do eclose have difficulty in fluid-filling of the wings. These studies show that this member of the calcium channel α_1 subunit gene family plays a nonredundant, vital role in larvae and adults.

MOLECULAR diversity in voltage-gated calcium channels has been revealed by pharmacological, electrophysiological, and gene cloning studies in both vertebrates (Catterall 1988, 1995; Bean 1989; Hess 1990; Tsien *et al.* 1991; Snutch and Reiner 1992; Hofmann *et al.* 1994) and invertebrates (Greenberg *et al.* 1989; Pelzer *et al.* 1989; Leung and Byerly 1991; Hille 1992; Skeer *et al.* 1992; Zheng *et al.* 1995; Smith *et al.* 1996). The well-studied vertebrate skeletal muscle L-type calcium channel is composed of five subunits α_1, α_2, β, γ, and δ with the α_1 subunit forming the ion selectivity pore through the membrane. Channel diversity arises from multiple genes encoding each calcium channel subunit (Perez-Reyes *et al.* 1990; Snutch *et al.* 1990; Hofmann *et al.* 1994; Catterall 1995), from alternative splicing (Perez-Reyes *et al.* 1990; Hui *et al.* 1991; Snutch *et al.* 1991; Hofmann *et al.* 1994; Dunlap *et al.* 1995), from RNA editing (Peixoto *et al.* 1997), from posttranslational modification (Nunoki *et al.* 1989; De Jongh *et al.* 1989) and from combinatorial association of different α_1 subunits with subtypes of non-α_1 subunits (Wei *et al.* 1991).

We have recently cloned and sequenced Dmca1D, a cDNA encoding a calcium channel α_1 subunit from Drosophila (Zheng *et al.* 1995). Dmca1D is most similar to the rat brain type D calcium channel α_1 subunit (Snutch *et al.* 1990) and is most strongly expressed in the developing larval and adult nervous systems (Zheng *et al.* 1995). Binding studies of calcium channel blockers

to Drosophila head membrane extracts (Greenberg *et al.* 1989) as well as electrophysiological recordings from reconstituted Drosophila brain membranes (Pelzer *et al.* 1989), from cultured Drosophila embryonic neurons (Leung and Byerly 1991), and from Drosophila larval muscle (Gielow *et al.* 1995) all provide evidence that in Drosophila, as in other organisms, multiple types of calcium channels are formed. Indeed, we have shown (D. Ren, H. Xu, D. F. Eberl, M. Chopra, and L. M. Hall, unpublished results) that calcium channel currents mediated by Dmca1D are sensitive to dihydropyridines (DHP), while another recently identified Drosophila calcium channel, Dmca1A, is structurally more similar to DHP-insensitive calcium channels (Smith *et al.* 1996; Peixoto *et al.* 1997).

The physiological significance of this diversity is unknown. Identification of mutations in individual calcium channel subunit genes is one approach to define the functional roles of each type of calcium channel. Here we show that the *l(2)35Fa* complementation group (Ashburner *et al.* 1990) represents the structural gene for the Dmca1D calcium channel α_1 subunit. Therefore, we name this locus *Dmca1D* (*Drosophila melanogaster* calcium channel α_1 subunit DHP-sensitive). We describe the developmental effects of mutations in *Dmca1D* on the organism and integrate these findings with our electrophysiological studies on this channel (D. Ren, H. Xu, D. F. Eberl, M. Chopra, and L. M. Hall, unpublished results).

MATERIALS AND METHODS

Genetic strains: All *Drosophila melanogaster* mutations and chromosomal aberrations in the 35E-F region were obtained

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from the laboratory of M. Ashburner and from the Bloomington Drosophila Stock Center. The *y w; Sb P{Δ2-3}99AB/ TM6* stock used for transformation was obtained from N. Perrimon. The *X7, X10*, and *AR66* alleles of *l(2)35Fa* (= *Dmca1D*) were induced on a common background chromosome, *b pr cn wx bw* (Ashburner *et al.* 1990). A fourth mutation induced on this background, *AR146*, mutates a nearby complementation group, *l(2)35Fd*. Because we did not have the original *b pr cn wx bw* stock, we used the *l(2)35Fd^AR146* chromosome, which should be unaltered in the *Dmca1D* gene, to represent the "wild-type" background for sequencing. Hemizygous *Dmca1D* region background DNA was obtained from *l(2)35Fd^AR146/ Df(2L)el18* flies because *Df(2L)el18* deletes *Dmca1D* but not *l(2)35Fd* (Figure 1A; Ashburner *et al.* 1990).

Isolation of genomic cosmid clone: The PB1 and PB2 probes (Figure 1B) were labeled with ^{32}P-dCTP (Megaprime random primer labeling kit; Amersham, Arlington Heights, IL) and used for high stringency screening of about 16,800 colonies from the *iso-1* Drosophila genomic cosmid library (J. Tamkun, University of California, Santa Cruz). PB1 is a 678-bp PCR fragment [coordinates 6885-7562 of Zheng *et al.* (1995)] from the 3′ end of the cDNA while PB2 is a 665-bp PCR fragment [coordinates 960-1624 of Zheng *et al.* (1995)] from the 5′ region.

Transformation: *P*-element-mediated transformation was carried out as described by Spradling (1986). Ca01 cosmid DNA was injected into *y w; Sb P{Δ2-3}99AB/ TM6* embryos and transformants were recognized by expression of the *white+* marker gene present in the cosmid vector.

Determination of lethal phase: Eggs were collected for 4 hr on yeasted apple juice agar plates and 200 eggs from each cross were counted and transferred to a fresh plate. After 30–36 hr, the larvae that hatched were transferred to standard food vials at a density of 25 per vial. Unhatched eggs were dechorionated with a 2-min treatment of 50% commercial bleach, rinsed, and covered in halocarbon oil for inspection under a compound microscope using brightfield and Nomarski optics.

Northern analysis: Northern blots were prepared and analyzed by standard methods (Sambrook *et al.* 1989). Poly(A$^+$) RNA was prepared by the guanidinium isothiocyanate-CsCl gradient method (Chirgwin *et al.* 1979) followed by one passage through an oligo(dT)-cellulose column. Poly(A$^+$)RNA (20 μg/lane) was electrophoresed on a 0.8% agarose gel containing 6.3% formaldehyde in 1 × MOPS buffer. Following capillary blotting (Nytran membrane; Schleicher & Schuell, Keene, NH) and UV crosslinking, the membrane was prehybridized for 4 hr at 42° in 50% deionized formamide, 5 × SSPE, 5× Denhardt's, 0.5% sodium dodecyl sulfate (SDS), 0.01% denatured salmon sperm DNA, and then hybridized with ^{32}P-labeled probe (2 × 10^6 cpm/ml) for 16 hr at 42°. Following high stringency washing, the blot was exposed to X-ray film for 7–21 days at −70°.

Mutation detection: For confirmation of the *X10* mutant change originally detected as a *Taq*I restriction site change, PCR (in 100 μl) was done with three different polymerases: AmpliTaq (Perkin Elmer, Norwalk, CT); Pfu (Stratagene, La Jolla, CA); HotTub (Amersham), and 0.1 μM of each primer {M13SH14A1B [coordinates 5159-5181 of Zheng *et al.* (1995)] and SP6SH18A1A [coordinates 6141-6163 of Zheng *et al.* (1995)]}. Thermal cycling conditions were: 35 cycles of 1 min at 95°, 1 min at 50°, and 90 sec at 72°, followed by 10 min at 72° and cooling to 4°. PCR products were extracted from a 1% agarose gel with GeneClean glass powder (Bio101, La Jolla, CA), digested with *Taq*I (GIBCO BRL, Grand Island, NY) for 1 hr at 65°, and analyzed on 2.5% agarose gel.

DNA sequencing: To sequence the *AR66* allele, double-stranded sequencing was performed on an Applied Biosystems Sequencer Model 373A (Applied Biosystems, Foster City, CA) using the dideoxy chain termination method either with fluo-

rescent dye-tagged primers (M13 or SP6) according to instructions supplied with the Taq Dye Primer Cycle Sequencing kit (Applied Biosystems, Inc.) or with fluorescent dye-tagged terminators. Purified PCR products from the *X10* mutant heterozygote were digested with *Acc*I and *Xba*I, subcloned into pBluescriptSK$^-$II (Stratagene), and colonies with different *Taq*I restriction digestion patterns were sequenced in the same way. The *AR66* mutant change detected by automated sequencing was confirmed by sequencing two PCR products, from different primer pairs that flank the change, with the CircumVent Cycle Sequencing kit (New England Biolabs, Beverly, MA) using the PCR primers and incorporation of ^{35}S-dATP.

Antibody staining of mutant embryos: Embryos homozygous for recessive lethal alleles (*X10, X7*, and *AR66*) of *Dmca1D* were analyzed with the following antibodies: Mab22C10 (provided by the laboratory of S. Benzer, CalTech, Pasadena, CA), MabBP102 and Mab1D4 (provided by the laboratory of C. Goodman, University of California, Berkeley), and anti-HRP (Cappel, Organon Teknika Corp., Durham, NC) and anti-cut (provided by L. Jan and Y. Jan, University of California, San Francisco). Embryos from heterozygous mutant/ *CyO, wg^len11* parents were collected overnight and prepared for staining by dechorionating in 2.5% sodium hypochlorite (50% bleach) for 5 min and rinsing with 0.1% Triton. Embryos then were permeabilized and fixed for 5 min in a mixture of 2.5% glutaraldehyde (Sigma Chemical, St. Louis, MO) and heptane (1:1). Vitelline membranes of embryos in the heptane layer were removed by adding 1–2 volumes of methanol and vortexing for 10 sec on moderate speed. Embryos were rehydrated in PTween (phosphate-buffered saline containing 0.1% Tween), and then stained for β-galactosidase with 0.2% X-gal in staining solution (10 mm sodium phosphate buffer pH 6.5, 150 mm NaCl, 1 mm MgCl$_2$, 3 mm K$_4$[Fe(CN)$_6$], 0.3% Triton) at 37° for approximately 30 min. All embryos then were reacted with primary antibodies (Mabs 22C10, BP102, and 1D4 diluted 1:10, anti-HRP diluted 1:10,000 and anti-cut diluted 1:1000) in PTween for 3 hr at room temperature. After washing in PTween, embryos were incubated in biotinylated horse anti-mouse or goat anti-rabbit secondary antibodies (Vector Laboratories, Burlingame, CA) for 3 hr at room temperature. Secondary antibodies were detected using a Vectastain Elite kit (Vector). Color was developed in a solution of 0.5% DAB (diaminobenzidine), 0.08% NiCl and 0.01% H$_2$O$_2$ in PTween. Embryos were mounted in 70% glycerol and homozygous mutant embryos of all stages were distinguished from sibling embryos by a failure to express β-galactosidase from an enhancer trap insertion in the *wg* gene on the *CyO, wg^len11* balancer chromosome.

RESULTS

Genetic and cytological mapping of *Dmca1D*: Genetic and cytological mapping of *Dmca1D*: To analyze the consequences of genetically disrupting the α$_1$ subunit Dmca1D, we used deletion mapping to determine whether any existing mutants corresponded to Dmca1D. We first used *in situ* hybridization to wild-type salivary gland polytene chromosomes with biotinylated probes from Dmca1D to determine the approximate map position and found that it hybridized to 35E3-F3 on the left arm of chromosome 2 (Zheng *et al.* 1995). Next, we extended this *in situ* hybridization analysis to deletions and other chromosomal aberrations to compare this map position with the extensive array of mutations that were previously mapped to this area (Ashburner *et al.* 1990). This analysis revealed a single complementation
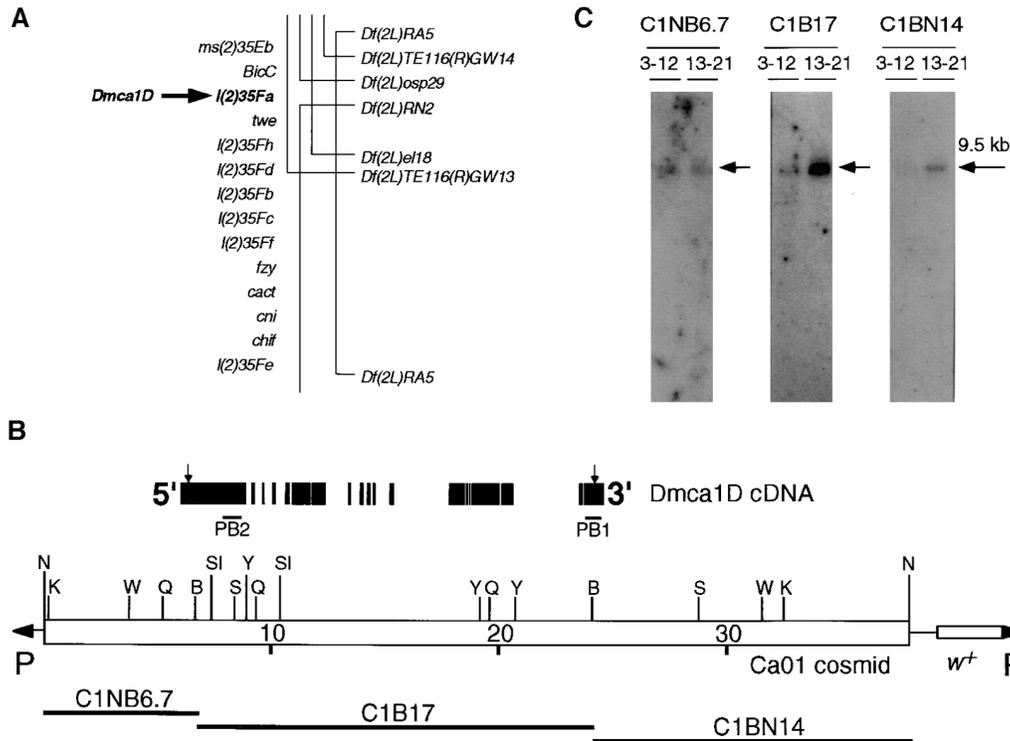
Figure 1.—Identification of *Dmca1D* mutant complementation group. (A) Genetic map of the *Dmca1D* region. Genes shown in the left column have been ordered by numerous deletions (Ashburner *et al.* 1990; Alphey *et al.* 1992), a subset of which are shown in the right column. The extent of each deletion is indicated by a vertical line, while the breakpoints are indicated by horizontal lines. Vertical lines connected to only one horizontal line represent deletions that extend beyond the limits of this figure. *Dmca1D* co-maps with the complementation group, *l(2)35Fa* (arrow), which we identify as the *Dmca1D* locus. (B) Genomic cosmid clone Ca01 that rescues *Dmca1D* mutations. The Ca01 cosmid used for *P* element-mediated transformation rescue of *l(2)35Fa* (*Dmca1D*) was isolated from a Drosophila genomic library using probes PB1 and PB2. Exons (black rectangles) in the Dmca1D cDNA clone are shown above the genomic clone. Arrows above the exon map indicate the proposed start and end of the ORF (Zheng *et al.* 1995). The positions of the *white* eye marker gene (*w⁺*) and the flanking *P* element ends (arrow heads) in the transforming construct are shown. C1NB6.7, C1B17, and C1BN14 are subclones of Ca01 used for probing Northerns shown in C. Restriction enzyme sites are: B = *Bam*HI; K = *Kpn*I; N = *Not*I; Q = *Xba*I; S = *Sal*I; SI = *Sst*I; W = *Nhe*I; Y = *Spe*I. The numbers within the long rectangle indicate the length in kilobases from the initial *Not*I site. (C) Embryonic Northern blots. mRNA from early (3–12 hr) or late (13–21 hr) embryos grown at 25° was used for replicate blots probed with the three subclones of Ca01 shown in B. A single transcript size was detected in late embryos (arrows).

group, *l(2)35Fa*, that showed the same mapping pattern as the Dmca1D clone (Figure 1A). Severe mutations (*X7* and *X10*) in this complementation group cause lethality at a late embryonic stage, while hypomorphic alleles (*AR66*) allow some adult escapers (Ashburner *et al.* 1990).

Calcium channels are known to be present in Drosophila embryos (Leung and Byerly 1991). Furthermore, developmental Northern analysis has shown that Dmca1D mRNA is most abundant in mid to late embryos (Zheng *et al.* 1995). These observations are consistent with an embryonic lethal phenotype for mutations in this α_1 subunit; thus *l(2)35Fa* is a good candidate for the Dmca1D structural gene.

Rescue of the *l(2)35Fa* lethality with a genomic cosmid clone: To determine whether the *l(2)35Fa* gene encodes Dmca1D, we used *P*-element-mediated transformation to test whether Dmca1D could rescue the lethal phenotype. We isolated a genomic cosmid clone, Ca01, that carries the entire Dmca1D coding region (19

kb) plus 6.63 kb of the 5′ upstream region (Figure 1B). Ca01 was injected into embryos that endogenously express the *P*-element transposase (Robertson *et al.* 1988). One resulting fly (Ca01.88) transmitted an integrated copy of the cosmid clone. Two derivatives (*Ca01.88C4* and *Ca01.88C7*) were stabilized in the following generations during removal of the transposase from the genotype. To test whether Ca01 rescues *l(2)35Fa* mutations, flies carrying the transforming cosmid in a mutant background were constructed. The transforming cosmid-bearing chromosome was crossed into a strain carrying the deletion *Df(2L)RA5*, which deletes a region including the *l(2)35Fa* gene (Figure 1A). Thus, flies heterozygous for the deletion and a mutant allele of *l(2)35Fa* will live only if the transformed Ca01 cosmid clone provides the function missing in the mutant. As shown in Table 1, both stable insertions (*Ca01.88C4* and *Ca01.88C7*) were fully able to rescue the lethality caused by each of the three mutant alleles of *l(2)35Fa* because we recovered w⁺ Cy⁺ flies in the

TABLE 1

Rescue of *l(2)35Fa* with the Ca01 cosmid

Cosmid insert[a]	*l(2)35Fa* allele[a]	Cy		Cy+	
		w+	w	w+	w
Ca01.88C4	X7	152	180	81	0
	X10	121	145	57	0
	AR66	150	180	81	16
Ca01.88C7	X7	178	167	97	0
	X10	262	251	139	0
	AR66	198	214	86	9
Ratio expected with complete rescue of a fully penetrant lethal allele		2	2	1	0

[a] Crosses were: *w;Df(2L)RA5/CyO; P{Ca01, w+}/+* crossed to *w;l(2)35Fa/CyO; +/+*, where *P{Ca01}* is either *Ca01.88C4* or *Ca01.88C7* and the *l(2)35Fa* allele is indicated in the table.

expected Mendelian frequencies. Some mutant survivors (w Cy+ flies) are produced by the leaky allele, *l(2)35Fa^{AR66}*, even in the absence of rescue by Ca01 (Table 1). The transformation results indicate that the gene product(s) that rescues *l(2)35Fa* is encoded within the Ca01 cosmid clone.

Embryonic transcripts encoded by the Ca01 cosmid clone: To determine how many different candidate embryonic transcripts were encoded by the rescuing Ca01 cosmid clone, three subclones (C1NB6.7, C1B17, and C1BN14) shown in Figure 1B were used to probe replicate wild-type embryonic Northern blots. Only one size class of message, 9.5 kb, was seen with each probe (Figure 1C). This is the size expected for Dmca1D (Zheng *et al.* 1995). Thus, a band the size of the Dmca1D message is the only one detected from the Ca01 genomic DNA region, providing further evidence that Dmca1D corresponds to the *l(2)35Fa* complementation group.

Identification of a premature stop codon in the *X10* allele: To determine whether the three *l(2)35Fa* alleles have alterations in the Dmca1D coding sequence, we used heteroduplex analysis with mutation detection enhancement (MDE) gels (Keen *et al.* 1991) to identify single base substitutions in heterozygote DNA. Heteroduplex analysis of genomic DNA including over 4 kb of the 7.5-kb open reading frame revealed an alteration in one allele, *l(2)35Fa^{X10}*, that suggested a *Taq*I site had been mutated. This lost *Taq*I site was confirmed by restriction enzyme digestion of a PCR fragment showing the appearance of a new, larger band (849 bp) in *l(2)35Fa^{X10}* heterozygotes but not in wild type (Figure 2A) and not in heterozygotes for the other mutant alleles (data not shown). Therefore, the *X10* allele of *l(2)35Fa* eliminates this *Taq*I site.

Subsequent genomic DNA sequencing showed that the wild-type *Taq*I site TCGA is mutated to TTGA (Fig-

ure 2B) in the *X10* allele, thereby changing an arginine CGA codon [R1800 of Zheng *et al.* (1995)] to a TGA stop codon. This change is in the cytoplasmic loop just following the IV-S4 transmembrane domain in the deduced Dmca1D protein (Figure 2E). This stop codon would produce a truncated protein that is missing the last two transmembrane domains (IV-S5 and -S6) and the cytoplasmic carboxy tail, which contains an EF hand motif, thought to bind Ca^{2+} (Babitch 1990; de Leon *et al.* 1995), part of the DHP-binding domain (Catterall and Striessnig 1992; Grabner *et al.* 1996), and the phenylalkylamine binding domain (Striessnig *et al.* 1990). Loss of these regions would produce a nonfunctional channel. Indeed, while deletions that remove 70% of the cytoplasmic carboxyl terminus of a cardiac calcium channel α_1 subunit lead to increased ionic currents, more extensive deletions abolish detectable current (Wei *et al.* 1994).

Identification of a missense mutation in the *AR66* allele: The ability to isolate homozygous mutant DNA from the surviving *AR66* homozygotes allowed us to test for the mutant change(s) by direct sequencing. Sequencing of most of the coding region with the exception of some of the larger introns revealed several changes, mostly silent, in *AR66* relative to the Dmca1D cDNA. All except one of these changes were found to be polymorphisms because they are present in all the mutant alleles and the wild-type chromosome from which they were generated (see materials and methods). The exceptional change, present only in *AR66* and not in *X7, X10*, nor in the wild type (Figure 2C), mutates a cysteine TGT codon [C629 of Zheng *et al.* (1995)] to a tyrosine TAT codon (Figure 2D). This cysteine is within the I-S1 transmembrane domain, closer to the extracellular side, of the deduced Dmca1D protein (Figure 2E). We have examined the functional significance of this residue in a separate study (D. Ren, H. Xu, D. F. Eberl, M. Chopra, and L. M. Hall, unpublished results) and find that the I-S1 segment is involved in determining the rate of channel activation and peak current.

The *Dmca1D* mutant phenotype: To investigate the lethal phenotype of *l(2)35Fa* alleles in more detail, we collected eggs from each mutant stock carrying the *CyO, wg^{len11}* balancer (see materials and methods) and followed them through development (Table 2). For the severe alleles, *X7* and *X10*, there are three classes of offspring in a roughly Mendelian ratio of 1:2:1. About half, the heterozygotes, live to adulthood. A quarter die as embryos with the characteristic wingless (wg) phenotype expected of the balancer homozygotes. The third class, representing the *l(2)35Fa* homozygotes, die uniformly at the late embryonic stage as pharate larvae with no gross morphological abnormalities. Nevertheless, the tracheae of *X7* and *X10* mutant embryos generally do not become gas-filled as they do in normal embryos. Gas-filling of tracheae may be associated with motor
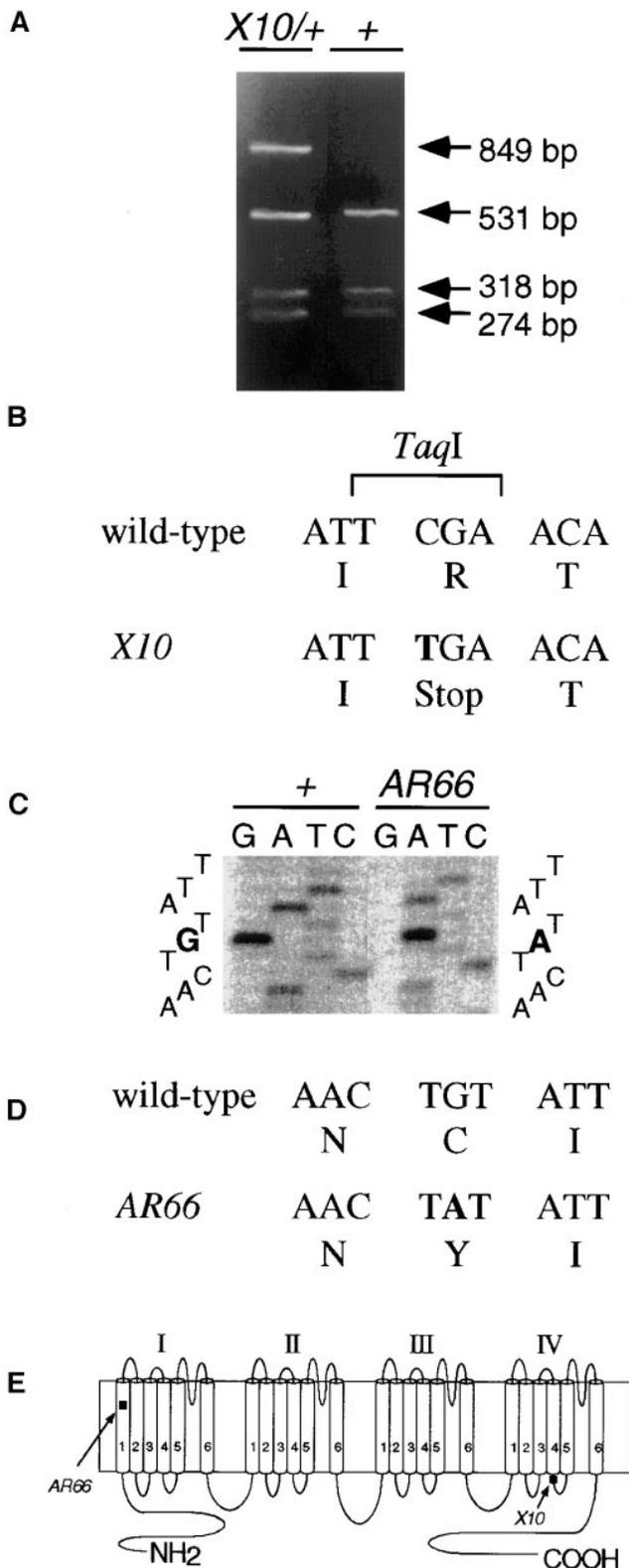
exertion or may be under control of the nervous system (see Manning and Krasnow 1993). The mutant embryos do move, but the movements are very weak, slow localized twitches, usually at the posterior end. Some peristaltic motion is visible in the gut, which is also very slow. Occasionally the embryos bend their heads, but movement of the cephalopharyngeal apparatus has not been seen. Pumping of the heart, which appears to require L-type calcium channel function (Gu and Singh 1995), is not seen in these mutant embryos. Certainly the vigorous writhing movements and extension of the mouthparts required for hatching are absent.

Conversely, flies homozygous for the weak allele, *AR66*, are all able to hatch as larvae, indicating that this allele retains partial function. In the uncrowded conditions used in this experiment 48% of the *AR66* homozygotes are able to eclose as adults (Table 2), although their development is delayed by 1–2 days at 25° relative to their siblings (data not shown). The wings of these homozygotes are usually unexpanded and many of the flies are found stuck in the food. The remaining 52% (Table 2) develop completely and usually manage to open the puparium but fail to eclose, so they die as pharate adults. No obvious differences could be detected in larval movements or heart rate between *AR66* homozygotes and heterozygotes.

To gain more insight into the nature of the hypomorphic *AR66* allele, we crossed it to the two strong alleles and to *Df(2L)RA5*, a deficiency for the region (Figure 1A). In all three cases (Table 2), the hatching and pupation frequencies are as high as from the *AR66* stock, indicating that the *trans*-heterozygous larvae are able to hatch and pupate. As with *AR66* homozygotes, there appears to be a major threshold during eclosion. Those that manage to eclose are usually found stuck in the

Figure 2.—Sequence changes in the calcium channel α_1 subunit in the *X10* and *AR66* alleles of *Dmca1D*. (A) A *Taq*I site is missing in the *X10* allele. Genomic DNA from mutant heterozygotes of the genotype *l(2)35Fa*^X10/+ or wild type (+ and wild-type refer to the "background" chromosome described in materials and methods) was amplified by PCR using three different polymerases (only AmpliTaq is shown). *Taq*I digestion produces an 849-bp fragment in mutant DNA that is cut into 531- and 318-bp pieces in wild type. (B) Premature stop codon in the *X10* allele. In wild-type DNA, the *Taq*I site is present but in the *X10* mutant allele, this site is destroyed by a C to T transition. This changes an Arg to a stop codon. (C) Missense mutation in the *AR66* allele. A sequencing gel using the CircumVent cycle sequencing kit (New England Biolabs) shows a substitution of A in the *AR66* allele for G in the wild-type background. The *X7* and *X10* alleles are unaltered (not shown). Because these sequence reactions are for the complementary strand, the gel image was rotated 180° to portray the coding strand. (D) Amino acid substitution in the *AR66* allele. A cysteine residue in the wild-type protein is substituted with a tyrosine residue in the *AR66* mutant protein. (E) Location of the *X10* and *AR66* mutant changes in the α_1 subunit. The α_1 subunit transmembrane configuration is shown diagramatically. The amino and carboxy termini are cytoplasmic. There are four repeats (I, II, III, IV) each of which is composed of six transmembrane domains (S1-S6) (Catterall 1988). Positions of the truncation in the *X10* allele and amino acid substitution in the *AR66* allele are indicated (black squares).

TABLE 2

Lethal phases of *l(2)35Fa* mutant alleles

	Eggs					Survivors		
			Dead					
Cross	Total	Fertilized	wg[a]	Late[b]	Hatched	Larvae	Pupae	Adults
$\dfrac{X7}{CyOen11}$	200	193	50	61	82	81	81	81[c]
$\dfrac{X10}{CyOen11}$	200	198	52	55	91	90	84	78[c]
$\dfrac{AR66}{CyOen11}$	200	194	55	3	136	134	130	126 (52)[d]
$\dfrac{AR66}{CyOen11} \times \dfrac{Df(2L)RA5}{CyOen11}$	200					147	140	135 (53)[d]
$\dfrac{AR66}{CyOen11} \times \dfrac{X7}{CyOen11}$	200					133	127	127 (2)[d]
$\dfrac{AR66}{CyOen11} \times \dfrac{X10}{CyOen11}$	200					149	148	148 (92)[d]

[a] The dead embryos with a wingless (wg) phenotype are homozygous for the *CyO, wg^{len11}* chromosome in which the *wingless* gene has been disrupted by an enhancer trap transposon insert.

[b] These dead embryos develop to a late stage, and appear structurally normal at a gross level. They are homozygous for the mutant allele indicated.

[c] These surviving flies are all Cy and are genotypically *l(2)35Fa/CyO, wg^{len11}*.

[d] These adults included both Cy and Cy^+ flies in a 2:1 ratio. The Cy^+ flies represent the critical class (mutant homozygotes) and include both flies that eclosed (these usually had unextended wings and were stuck in the food) and flies that did not fully eclose but died as pharate adults. In parentheses is the percent of Cy^+ flies that died as pharate adults.

food with unexpanded wings; those that fail usually open their puparia but die before being able to crawl out.

AR66/Df(2L)RA5 flies appear to be indistinguishable from *AR66* homozygotes (Table 2) in that about half of these flies die as pharate adults. *AR66/X7* flies, however, are almost all able to escape the puparium while very few *AR66/X10* flies do so (Table 2). These results were reproduced in a second independent experiment (data not shown). This may reflect differences in the nature of the *X10* and *X7* alleles.

To determine whether genetic disruption of this calcium channel subunit compromises nervous system formation, various antibodies were used to examine the developing nervous systems of mutant embryos (Figures 3 and 4). Mab22C10 stains subsets of neurons and axons in the central nervous system (CNS) and peripheral nervous system (PNS) (Fujita *et al.* 1982) (Figure 3, A–D); MabBP102 labels CNS axons (Seeger *et al.* 1993) (Figure 3, E and F); Mab1D4 was generated against the cytoplasmic domain of transmembrane forms of fasciclin II and recognizes subsets of neurons and axons in the CNS (Seeger *et al.* 1993) (Figure 4, A and B) and motorneuron growth cones and axons in the PNS (Van Vactor *et al.* 1993) (Figure 4, C and D). In addition to the antibodies shown in the figures, we have also used anti-HRP (data not shown), which stains neuronal membranes (Jan and Jan 1982), and anti-cut (data not shown), which labels nuclei of many CNS cells and all

external sensory organ cells in the PNS (Blochlinger *et al.* 1990). With one exception, in mutant embryos of the three *l(2)35Fa* alleles tested, no abnormalities were detected with any of these antibodies, indicating that at this level of analysis the mutant embryonic nervous systems appear morphologically normal.

The exception is that, in the CNS of a few (about 20%) homozygous *AR66* embryos, neurons in the longitudinal tracts appeared to stall at some of the commissures, forming nodular growths (Figure 4B) rather than the normal smooth longitudinal tracts seen in the strong *X10* mutant (Figure 4A) and in wild-type embryos (Seeger *et al.* 1993) (not shown). In a similar fraction of the *AR66* homozygous embryos, the motorneurons in the SNb branch of the developing PNS also show stalling (Figure 4D) at stage 17 when the SNb has normally already formed the three characteristic muscle attachments (Figure 4C) into the target muscles (Van Vactor *et al.* 1993). The observations that these defects are low in frequency and that they do not appear in *X10*, the strong allele tested, suggest that they may be caused by homozygosity of another lesion on the *AR66* chromosome unrelated to the *Dmca1D* locus. Alternatively, they could be generated by the altered channel properties caused by the *AR66* missense mutation (D. Ren, H. Xu, D. F. Eberl, M. Chopra, and L. M. Hall, unpublished results).

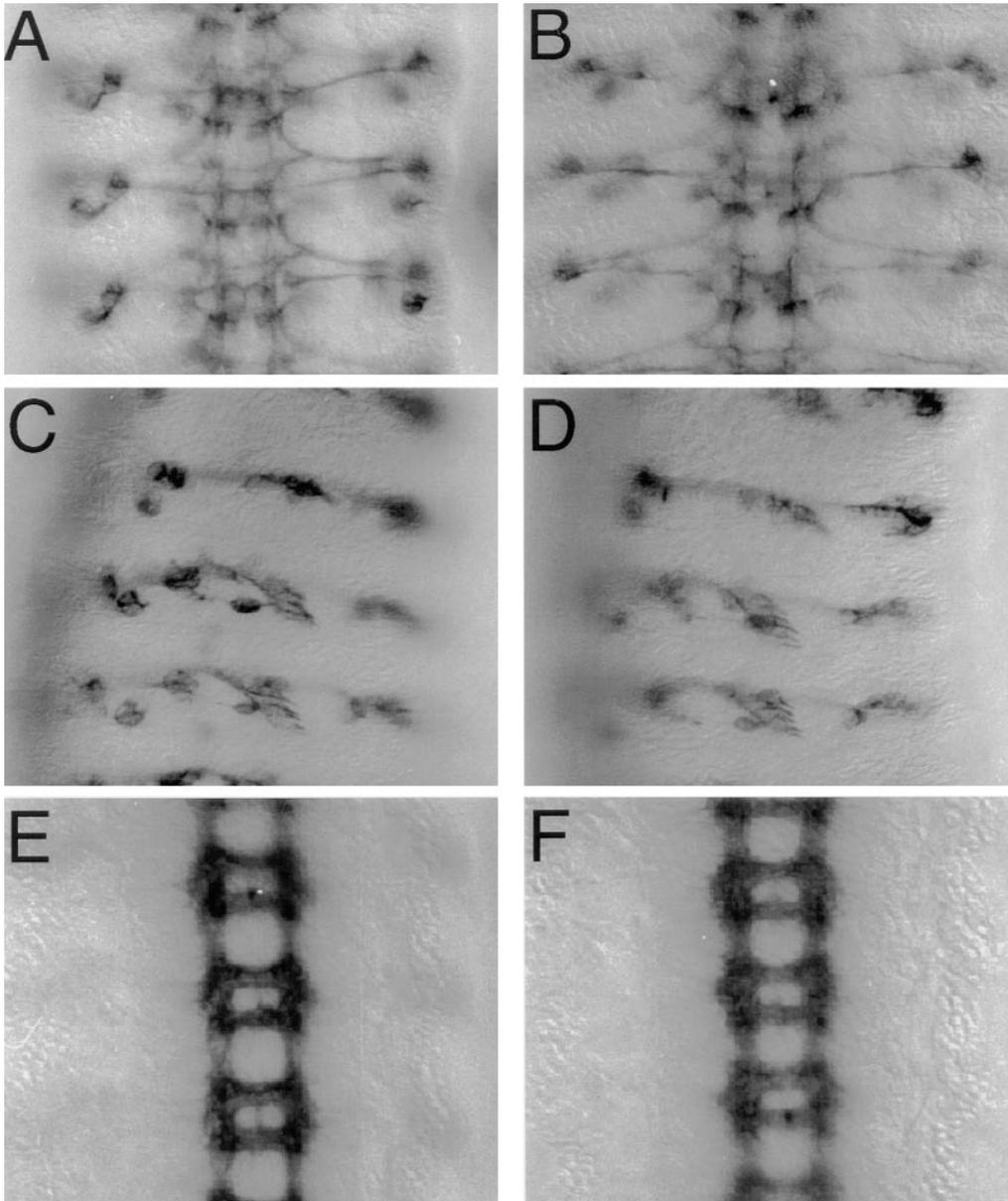We have also tested for maternal effects that might

Figure 3.—Antibody staining of *X10* mutant embryonic nervous systems. Heterozygous control (A, C, E) and sibling homozygous *X10* mutant (B, D, F) embryos stained with antibodies that reveal the structure of various aspects of the nervous system. Mab22C10 recognizes a subset of neurons and axon tracts in the CNS (A, B) and neurons of sensory chordotonal organs and nerve branches in the PNS (C, D). MabBP102 recognizes a surface antigen on all CNS axons (E, F). All embryos are oriented with anterior at the top. A, B, E, and F are ventral views, while in lateral views (C, D), ventral is to the left.

be associated with these lethal mutations. Many lethal mutations with apparently late effects have much earlier requirements if the normal gene products provided maternally to the egg are eliminated (Perrimon *et al.* 1984). We tested for maternal effects by generating homozygous mutant ovaries in heterozygous mothers using the "FLP-DFS technique" (Chou and Perrimon 1996). We have found no evidence for maternal effects associated with the three *l(2)35Fa* alleles tested (data not shown).

DISCUSSION

The *l(2)35Fa* gene (*Dmca1D*) encodes the Dmca1D channel: We have presented several lines of evidence indicating that *l(2)35Fa* is the structural gene encoding the calcium channel α_1 subunit Dmca1D. First, it is the only candidate complementation group that co-maps with Dmca1D, even after extensive mutagenesis screens in a number of laboratories (Ashburner *et al.* 1990). Second, we rescued *l(2)35Fa* with a genomic cosmid that encodes the calcium channel α_1 subunit and demonstrated that the α_1 subunit transcript is the only one detectable in embryos by probes from this cosmid. Third, we have identified within the calcium channel open reading frame a premature stop codon in the *X10* mutant allele and a missense mutation in the *AR66* allele of *l(2)35Fa.*

Additional electrophysiological studies on the *AR66* mutation (D. Ren, H. Xu, D. F. Eberl, M. Chopra, and L. M. Hall, unpublished results) demonstrated reduced DHP-sensitive calcium channel current density with slower activation kinetics in third instar larval muscles. In addition, the embryonic lethality of *l(2)35Fa* is consistent with our earlier observation that flies fed with
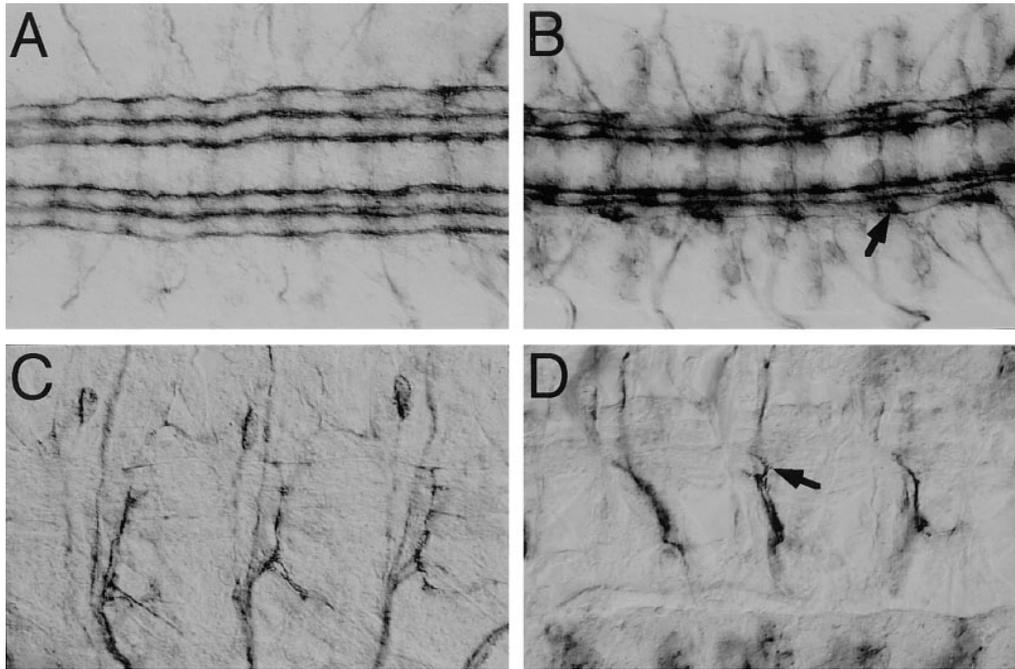
Figure 4.—CNS and PNS phenotypes in filleted *X10* and *AR66* mutant embryos. Mab1D4, generated against fasciclin II, stains longitudinal axon tracts in the CNS (A–B) and, in the PNS, major motorneuron axon branches innervating the ventral musculature (C–D). The longitudinal axon tracts are normal in *X10* homozygotes (A, C) but approximately 20% of homozygous *AR66* embryos show stalling of neurons in the CNS (arrow in B). In the SNb motorneuron branch, while pathfinding and muscle innervation is normal in *X10* homozygotes (C), in about 20% of *AR66* homozygotes at the same developmental stage (stage 17) these neurons have not (yet) innervated their target muscles (D). These defects in *AR66* embryos may be caused by the altered properties of the AR66 channel or by a lesion elsewhere on the *AR66* chromosome (see text).

the calcium channel blocker verapamil show dose-dependent lethality (Hall *et al.* 1994). Those flies that do survive at moderate doses of verapamil show delayed development, consistent with delayed development of *AR66* mutant flies. Furthermore, late embryonic lethality is consistent with the peak of α_1 subunit mRNA expression detected with Dmca1D probes in late embryos (Zheng *et al.* 1995). Taken together, these results constitute overwhelming evidence that *l(2)35Fa* encodes Dmca1D. Based on these results and arguments, we propose to rename the *l(2)35Fa* locus *Dmca1D.*

Role of *Dmca1D* in embryos and adults: These studies demonstrate that *Dmca1D* is first required in the developing embryo and later in late pupal stages, both times when Dmca1D mRNA is expressed at peak levels (Zheng *et al.* 1995). There is no maternal effect and we have found no obvious structural abnormalities in the mutant embryonic CNS or PNS that we could attribute to the Dmca1D mutations. These observations, together with those of abnormal movement of the mutant embryos, suggest that the embryonic defect is primarily physiological rather than a gross disruption of nervous system development. Our finding that *AR66* larvae have defects in muscle calcium channel currents is consistent with this interpretation. Thus, function of *Dmca1D* appears to be required for the muscle contractions (or their neuronal modulation) for the pharate larva to make

proper hatching movements. *Dmca1D* also appears to be necessary for gas-filling of the tracheae, either actively or, as more classically thought, passively, through metabolic exertion or physical movement (see Manning and Krasnow 1993). This could be resolved by a detailed mapping of the cells which require *Dmca1D* function for the gas-filling process. *Dmca1D* may also be involved in modulating contraction in the gut and heart.

While these pharate larval movements are disrupted by the strong mutations *X7* and *X10*, they are not detectably altered by the weak allele, *AR66.* Larvae expressing this mutation are able to hatch normally, and are behaviorally indistinguishable from their wild-type siblings throughout the larval period, displaying the first abnormalities as late pupae. These abnormalities are manifest as a delay in development, difficulty in eclosion and disturbed fluid-filling of the wings for proper expansion. The mechanistic significance of the *AR66* mutation for the larva appears to be that the slower activation and reduced current density of the *AR66* channel provides reduced larval calcium channel activity, but still enough to meet the larval requirements. However, *AR66* mutant channel function clearly is not sufficient to fulfill the adult requirements of *Dmca1D.*

As in the embryo, it is possible to explain the pharate adult defects as either muscle or neuronal defects. First, the process of eclosion from the puparium requires

vigorous muscular activity. The ptilinum, or inflatable head, of the pharate adult is cyclically inflated and deflated, allowing it to wedge into the anterior part of the puparium in order to pry it open (Laing 1935; Crossley 1978). Inflation of the ptilinum is achieved by the contraction of the supercontracting abdominal muscles (of larval origin; they degenerate within a few days of eclosion), forcing the hemolymph into the head. Deflation occurs by contraction of the ptilinal retractor muscles in the head (these also degenerate in a couple of days). Second, the process of wing expansion in the first hour after eclosion is based on pharyngeal muscle activity. Upon eclosion the fly begins to swallow air until a large bubble has accumulated in the midgut (Eidmann 1924; Fraenkel 1935). This increase in gut volume greatly increases the pressure of the hemolymph, distending the entire fly and forcing a steady stream of hemolymph into the wings to inflate them (Lagueux and Perron 1973). Thus, all the phenotypes we see, from defects in embryonic muscular contractions in strong mutants, to defects in third instar larval muscle electrophysiology (D. Ren, H. Xu, D. F. Eberl, M. Chopra, and L. M. Hall, unpublished results), to defects in eclosion and wing expansion in pharate adults carrying the weaker mutation, are consistent with defects in muscle contraction, though neuronal modulation of the contractions could also be affected.

Use of heteroallelic mutant combinations revealed differences in the proportion of mutant pupae that die as pharate adults suggesting differences in the nature of the *X7* and *X10* alleles. One possibility is that the *X10* truncated protein sequesters some of the other subunits, leaving a smaller pool to associate with the AR66 channel. This would result in the *AR66/X10* combination being more detrimental than *AR66* alone. The X7 channel, conversely, may have none of the embryonic functional activity so that *X7* homozygotes are embryonic lethal, but may have residual pupal activity so that in *AR66/X7* heterozygotes the AR66 channel fulfills the embryonic requirement and the X7 channel gives a very small pupal boost. Identification of the mutant change in the *X7* allele may help to address this question.

A few calcium channel α₁ subunit mutations have been identified in other organisms. The *muscular dysgenesis* (*mdg*) mutation in a mouse skeletal muscle calcium channel α₁ subunit gene causes lethality (Chaudhari 1992), and mutations in skeletal muscle calcium channel α₁ subunit (DHP receptors) in humans cause hypokalemic periodic paralysis (Ptácek *et al.* 1994). Mutations in a nematode calcium channel α₁ subunit gene, expressed in specific neurons and the body wall muscle, indicate that this calcium channel plays a modulatory role in adaptation to dopamine and serotonin (Schafer and Kenyon 1995) in certain neurons. Mutations in the P/Q-type neuronal calcium channel α₁ₐ subunit are responsible for absence epilepsy and ataxia in the *tot-*

tering mouse mutant strains (Fletcher *et al.* 1996). Mutations in the human brain CACNL1A4 calcium channel α₁ₐ gene are associated with familial hemiplegic migraine and episodic ataxia type-2 (Ophoff *et al.* 1996).

Drosophila calcium channel α₁ subunit gene family: Gene cloning studies in a variety of species have shown that there are multiple genes encoding structurally similar α₁ subunits (Perez-Reyes *et al.* 1990; Snutch *et al.* 1990; Hofmann *et al.* 1994; Catterall 1995). Similarly, Drosophila has at least two distinct genes encoding α₁ subunits: *Dmca1D* on the second chromosome (Zheng *et al.* 1995; this work) and *Dmca1A* on the *X* chromosome (Smith *et al.* 1996; Peixoto *et al.* 1997). These two α₁ subunits encoded by different genes are not functionally redundant since each of these genes can be mutated to lethality. Alleles of the *Dmca1A* gene have also been identified that affect vision and song production (Smith *et al.* 1996), processes that have not been associated with *Dmca1D.*

Test system for function of *Dmca1D* isoforms: We have found evidence for extensive alternative splicing among *Dmca1D* transcripts (Zheng *et al.* 1995). Now that *Dmca1D* mutations have been rescued with a genomic construct, and mutant phenotypes have been defined, transformation studies can be used to determine the biological significance of variously spliced forms using different cDNAs under the control of the endogenous promoter. Such analyses will define the contribution of alternative splicing to the physiological heterogeneity of Drosophila calcium channels (Pelzer *et al.* 1989; Leung and Byerly 1991).

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