Synaptic Calcium-Channel Function in *Drosophila*: Analysis and Transformation Rescue of Temperature-Sensitive Paralytic and Lethal Mutations of *Cacophony*

Fumiko Kawasaki, Stephen C. Collins, and Richard W. Ordway

Department of Biology, The Pennsylvania State University, University Park, Pennsylvania 16802

Voltage-gated calcium channels play a key role in chemical synaptic transmission by providing the calcium trigger for regulated neurotransmitter release. Genes encoding the primary structural subunit, $\alpha 1$, as well as accessory subunits of presynaptic calcium channels have now been identified in a variety of organisms. The *cacophony (cac)* gene in *Drosophila*, also known as *nightblind A*, encodes a voltage-gated calcium-channel $\alpha 1$ subunit homologous to vertebrate $\alpha 1$ subunits implicated in neurotransmitter release. A recent genetic screen in our laboratory isolated *cac^{TS2}*, a conditional *cac* mutant exhibiting rapid paralysis at elevated temperatures. This mutant has allowed synaptic electrophysiology after acute perturbation of a specific calcium-channel gene product, demonstrating that *cac* encodes a primary calcium channel functioning in neurotransmitter release. Here we report the molecular lesion in *cac^{TS2}*, a missense mutation within a calcium-dependent regulatory domain of the $\alpha 1$ subunit, as well as phenotypic rescue of temperature-sensitive and lethal *cac* mutations by transgenic expression of a wild-type *cac* cDNA. Notably, rescue of rapid, calcium-triggered neurotransmitter release was achieved by neural expression of a single cDNA containing a subset of alternative exons and lacking any conserved synaptic-protein interaction sequence. Possible implications of these findings are discussed in the context of structure–function studies of synaptic calcium channels, as well as alternative splicing and mRNA editing of the *cac* transcript.

Key words: synapse; calcium channel; Drosophila*; temperature-sensitive; alternative splicing; SYNPRINT*

Calcium channels implicated in neurotransmitter release are composed of a primary structural subunit, $\alpha 1$, as well as β, $\alpha 2/\delta$, and possibly γ subunits (for review, see Catterall, 1998). The $\alpha 1$ subunit alone may form voltage-gated calcium channels in heterologous expression systems; however, coexpression of accessory subunits is thought to more closely reproduce the function and regulation of endogenous channels (for review, see Walker and De Waard, 1998; Ikeda and Dunlap, 1999). The $\alpha 1$ subunit polypeptide is organized into four repeating domains, each including six transmembrane segments (Fig. 1*A*). Vertebrate genes encoding primary $\alpha 1$ subunits functioning in neurotransmitter release include $\alpha 1A$ ($Ca_v 2.1$) and $\alpha 1B$ ($Ca_v 2.2$), which encode P/Q- and N-type channels, respectively. Both of these channel types have been localized to presynaptic terminals (for review, see Stanley, 1997; Catterall, 1998), and the pharmacology of these channels when expressed in heterologous systems is similar to that observed for neurotransmitter release.

Synaptic calcium channel diversity

The structural diversity of presynaptic calcium channels is provided by different $\alpha 1$ subunit genes, as well as assembly of $\alpha 1$ with different combinations of accessory subunits. In contrast to the closely related $\alpha 1$ subunit genes encoding vertebrate presynaptic calcium channels, *cacophony (cac)* appears to be the only homol-

ogous gene in *Drosophila* (Smith et al., 1996; Littleton and Ganetzky, 2000). Fly genes encoding L-type (Gielow et al., 1995; Zheng et al., 1995; Ren et al., 1998) and T-type calcium channels, as well as each of the known accessory subunits, have been identified as well (Littleton and Ganetzky, 2000).

Another mechanism for generating presynaptic calcium-channel variants involves alternative splicing of $\alpha 1$ subunit mRNAs. This process acts on mammalian $\alpha 1A$ and $\alpha 1B$ transcripts to produce a variety of mRNAs encoding different $\alpha 1$ subunit proteins (Lin et al., 1997, 1999; Bourinet et al., 1999; Hans et al., 1999; Krovetz et al., 2000; Pan and Lipscombe, 2000). Similarly, alternative splicing of the *cac* $\alpha 1$ subunit mRNA contributes to calcium-channel diversity in *Drosophila* (Smith et al., 1996, 1998b; Peixoto et al., 1997). Several alternative exons have been described in *cac*, including two mutually exclusive exon pairs, IS4a/IS4b and I-IIa/I-IIb, as well as a 3, 6, or 9 bp insertion adding one to three amino acids to the IVS3–IVS4 loop (Smith et al., 1996; results reported here). The latter splicing site appears to be conserved in mammalian $\alpha 1$ subunit genes. At the equivalent position within the $\alpha 1A$ and $\alpha 1B$ transcripts, inclusion of a 6 bp alternative exon adds two amino acids to the IVS3–IVS4 loop and alters functional properties of the channels (Lin et al., 1997, 1999; Bourinet et al., 1999; Hans et al., 1999; Krovetz et al., 2000). Additional mRNA splicing variants of *cac* are reported in the present study.

Structural diversity of synaptic calcium channels may also be generated through editing of $\alpha 1$ subunit mRNAs. A-to-I mRNA editing results in conversion of adenosine residues to inosine, which may behave as guanosine during translation (for review, see Reenan, 2001). Editing of the *cac* transcript by this mechanism alters the *cac* coding sequence at a number of positions (Smith et al., 1998a; Palladino et al., 2000) (see Figure 3); however, the

A Presynaptic Voltage-Gated Ca^{2+} Channel α1 Subunit
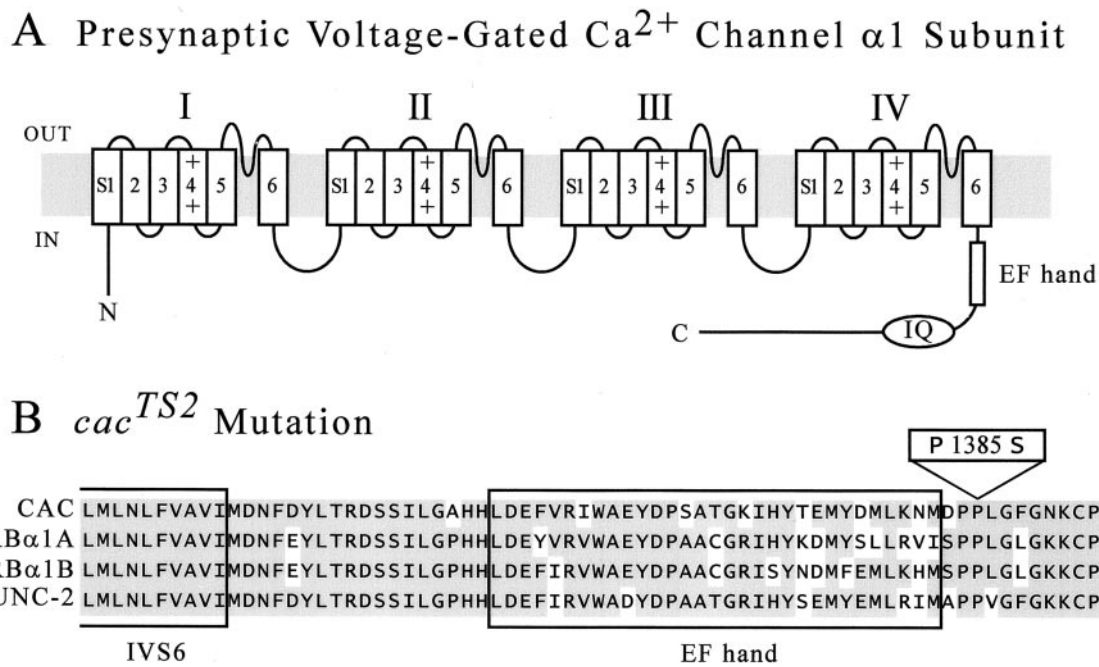

B *cac^{TS2}* Mutation

Figure 1. A missense mutation in *cac^{TS2}*. *A*, Presynaptic voltage-gated calcium-channel α1 subunit topology. *Shading* represents the plasma membrane. *Roman numerals* refer to the four repeating domains, each including six transmembrane segments (S1–S6). A charged S4 segment is thought to serve as a voltage sensor. An intramembranous "P loop" between the S5 and S6 segments of each repeat contributes to formation of the channel pore. Both the N and C termini of the α1 subunit are intracellular, and the four repeats are linked by three major intracellular loops. Also represented are calcium-binding (*EF hand*) and calmodulin-binding (*IQ*) domains within the C-terminal cytoplasmic tail. *B*, The *cac^{TS2}* mutation. Alignment of CAC with related calcium-channel α1 subunit polypeptide sequences is shown. Amino acid identities with CAC are *shaded*. *Boxed sequences* correspond to the EF hand and a portion of the IVS6 transmembrane segment. The *cac^{TS2}* mutation, P1385S, maps to an invariant proline residue adjacent to the EF hand. The aligned sequences correspond to CAC (U55776), rat brain α1A and α1B (M64373 and M92905, respectively), and *Caenorhabditis elegans* UNC-2 (U25119).

functional consequences of this editing remain to be determined. To date, editing of calcium-channel transcripts has been described only in *Drosophila*; however, editing of other mammalian ion-channel mRNAs and its functional consequences have been reported previously (Higuchi et al., 2000; Reenan, 2001).

Mechanisms of calcium-channel regulation

The gating of presynaptic calcium channels is regulated by several mechanisms, including direct α1 subunit interactions with G-proteins, calcium/calmodulin, and components of the neurotransmitter release apparatus. Inhibition of neurotransmitter release by G-protein-linked receptor agonists occurs through direct interactions between the calcium-channel α1 subunit and βγ subunits of heterotrimeric G-proteins (De Waard et al., 1997; Dunlap, 1997; Zamponi et al., 1997; Mirotznik et al., 2000; Colecraft et al., 2001). Regulation by G-proteins is antagonized by protein kinase C-mediated phosphorylation of the α1 subunit (Zamponi et al., 1997; Herlitze et al., 2001), which has also been reported to increase basal calcium current (Yang and Tsien, 1993). Another regulatory mechanism involves direct binding of calcium/calmodulin to the IQ motif within the C-terminal cytoplasmic domain of the α1 subunit and is thought to mediate calcium-dependent channel gating, including facilitation and inactivation (Lee et al., 1999; DeMaria et al., 2001; Erickson et al., 2001). An EF hand calcium-binding motif within the same C-terminal region of the α1 subunit may also contribute to calcium-dependent inactivation (Peterson et al., 2000). Finally, interaction of presynaptic calcium-channel α1 subunits with syntaxin, a core protein of the neurotransmitter release apparatus, has been shown to regulate channel gating (Bezprozvanny et al.,

1995, 2000; Degtiar et al., 2000) and also to promote regulation by G-proteins (Stanley, 1997; Jarvis and Zamponi, 2001; Lü et al., 2001). Binding of syntaxin and several other synaptic proteins to calcium channels led to identification of a synaptic-protein interaction (SYNPRINT) domain within the intracellular loop linking domains II and III of α1A (P/Q-type) and α1B (N-type) subunits (for review, see Sheng et al., 1998). This domain is proposed to mediate fast coupling of calcium influx to synaptic vesicle fusion by tethering calcium channels and the release apparatus and by participating in calcium-channel regulation (Mochida et al., 1996; Sheng et al., 1998; Wu et al., 1999; Zhong et al., 1999). Although our previous work has shown that *cac*-encoded calcium channels function in fast, calcium-triggered neurotransmitter release (Kawasaki et al., 2000), no sequence homologous to known calcium-channel synaptic-protein interaction domains is present in *cac* or elsewhere in the fly genome (Kawasaki et al., 2000; Littleton and Ganetzky, 2000). These findings suggest either a novel synaptic-protein interaction domain or an alternative mechanism for the fast coupling of calcium influx to synaptic vesicle fusion.

The central importance of presynaptic calcium channels has motivated genetic analysis to investigate the *in vivo* functions of specific calcium-channel proteins at native synapses (Schafer and Kenyon, 1995; Dove et al., 1998; Lorenzon et al., 1998; Jun et al., 1999; Saegusa et al., 2000; Ino et al., 2001). This presents several challenges, including the long-term compensatory changes that may occur in null or hypomorphic mutant animals (Jun et al., 1999; Saegusa et al., 2000; Ino et al., 2001). Thus, temperature-sensitive (TS) paralytic mutants of *Drosophila* provide an impor-

tant and complementary tool allowing acute perturbation of specific gene products for analysis of the molecular mechanisms underlying physiological processes. Our previous genetic (Dellinger et al., 2000) and electrophysiological (Kawasaki et al., 2000) studies of the TS paralytic mutant cac^{TS2} demonstrated that *cac* encodes a primary calcium-channel $\alpha 1$ subunit functioning in neurotransmitter release. Here we report characterization of the molecular lesion underlying the TS paralytic and synaptic phenotypes of cac^{TS2}, as well as rescue of *cac* mutants by transgenic expression of a specific *cac*-encoded $\alpha 1$ subunit. These studies further characterize a TS paralytic calcium-channel mutant and further define the molecular determinants governing *in vivo* function of presynaptic calcium channels.

MATERIALS AND METHODS

Fly stocks

cac^{TS2} was from our laboratory stock collection. The *cac* lethal mutants $l(1)L13^{20-3}$ and $l(1)L13^{HC129}$ were generously provided by Jeffrey C. Hall (Brandeis University, Waltham, MA). These were established in the following balanced lines: $l(1)L13^{20-3}/In(1)FM7i$, y^{93j} sc^8 w^1 oc^1 ptg^1 B^1 $P\{w^{+mC} = ActGFP\}JMR3$ and $l(1)L13^{HC129}/In(1)FM7i$, y^{93j} sc^8 w^1 oc^1 ptg^1 B^1 $P\{w^{+mC} = ActGFP\}JMR3$. The X-linked *elav-GAL4* enhancer trap line, $P(w^+)$ $elav^{C155}$, was obtained from the Bloomington Stock Center (Indiana University, Bloomington, IN). This driver will be referred to as *elav-GAL4*. *elav-GAL4* cac^{TS2}, *elav-GAL4* $l(1)L13^{20-3}$, and *elav-GAL4* $l(1)L13^{HC129}$ recombinant chromosomes were generated in our laboratory. Wild-type (WT) flies were *Canton S*.

Molecular analysis

Preparation of head RNA. Fifty flies were placed in a microcentrifuge tube, frozen in liquid nitrogen, and vortexed to remove the heads, which were collected and homogenized in 3 M LiCl and 6 M urea for total RNA preparation by conventional methods.

Reverse transcriptase PCR. Total RNA from 50 heads was used for first-strand cDNA synthesis by conventional methods using Moloney murine leukemia virus reverse transcriptase (RT) (Invitrogen, Carlsbad, CA) and random oligonucleotide primers (Invitrogen). A previously reported *cac* sequence (Smith et al., 1996) was used to design primers for RT-PCR using the head cDNA preparations as a template. The entire *cac* open reading frame (ORF) was amplified in three segments using six primers. Two different primer sets were used to generate slightly different PCR fragments for sequencing (primer set 1) and cloning (primer set 2). Primer set 1 included the following primer pairs (forward and reverse primers in a pair are separated by a slash): *cac*11-catcaacaggactccttagg/ *cac*21-caccaccctgagatatgatg; *cac*12-cccgatagcactgttgactg/*cac*22-gaattttccaccgtacctag; and *cac*13-ggcacttccctatgtctgtt/*cac*23-cctgatgctataccagatc. Primer set 2 included the following: *cac*11-catcaacaggactccttagg/*cac*22B-cggtgaatcccatgttaatg; *cac*12-cccgatagcactgttgactg/*cac*22-gaattttccaccgtacctag; and *cac*12A-ccaaccaatcccatacgacg/*cac*23-cctgatgctataccagatc. For direct sequencing of PCR products, PCRs were performed with *Taq* polymerase (PGC Scientific, Gaithersburg, MD), whereas Pfu polymerase (Stratagene, La Jolla, CA) was used in reactions for cloning.

Sequencing. Direct sequencing of PCR products and sequencing of *cac* clones was performed at the Penn State Nucleic Acids Facility. Sequencing primers for each of the three *cac* ORF fragments were designed on the basis of a previously reported *cac* sequence (Smith et al., 1996). Because cac^{TS2} was isolated as an extragenic enhancer of *comatose*^{ST53} (Dellinger et al., 2000), the cac^{TS2} mutation was identified by comparing the *cac* ORF sequence from cac^{TS2} and the *comatose*^{ST53} parent chromosome. Any apparent sequence differences were re-examined in independent RNA preparations. With respect to the published *cac* coding sequence (U55776), three silent polymorphisms were detected in *cac* ORF sequences; these were present in both cac^{TS2} and the parent *comatose*^{ST53} line. Sequence alignments were performed by the Clustal method, using the Megalign feature of the Lasergene Software Package (DNAStar, Madison, WI).

Cloning. Each of the three *cac* ORF fragments generated with primer set 2 was cut at unique restriction sites and cloned separately into the pBluescript SK$^-$ vector. These overlapping segments (Fig. 2) were then assembled into a single clone containing a complete ORF, which was subsequently shuttled into the *Kpn*I and *Not*I sites of the transformation vector, pUAST (Brand and Perrimon, 1993). Clones of ORF segments B
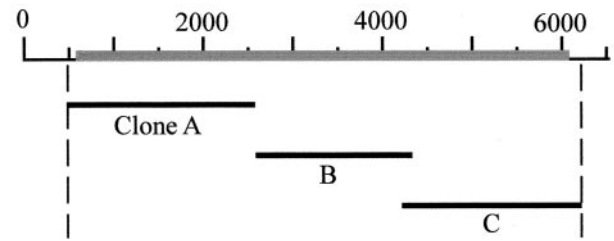

Figure 2. Three overlapping cDNA clones assembled into a clone containing the entire *cac* ORF. Details regarding the generation and cloning of these segments are provided in Materials and Methods. Nucleotide positions (in base pairs) correspond to those of the *cac* cDNA sequence (U55776). The ORF is represented as a *gray bar*.

and C (Fig. 2) were toxic to several different strains of bacteria. These could be propagated successfully in JM109 cells (Promega, Madison, WI); however, only small colonies were obtained, and liquid cultures grew poorly. The presence of specific splice variants among the population of clones for each segment was determined by sequencing and restriction mapping. As described in Results, two novel alternative exons of 12 and 60 bp were found in the region of the transcript encoding the C-terminal cytoplasmic tail of the $\alpha 1$ subunit. These were identified as contiguous exons in the *cac* gene and have the following nucleotide sequences (60 bp, CGGAAGAAGCTGGAGCACGATGATGAGCA-TAAATATAGCCCAACGGCAGTCGAGGAGCCG; 12 bp, AACT-GGAAGGAG).

Transgenic lines were generated essentially as described previously (Karess and Rubin, 1984). Briefly, the transformation construct *UAS-cac1* in pUAST was prepared for injection by column purification (Plasmid Maxi Kit; Qiagen, Valencia, CA). DNA (0.8 $\mu g/\mu l$) was injected into the posterior pole of w^{1118} embryos before cellularization. Coinjection of the pπ25.7wc plasmid (0.2 $\mu g/\mu l$) provided transient expression of the P element transposase. The w^+ marker carried by pUAST was used to select transformed progeny.

Transformation rescue

Rescue data were obtained using the third chromosome insertion line *UAS-cac1 256B*. For rescue of both TS and lethal *cac* phenotypes, eight independent *UAS-cac1* insertion lines were tested; seven produced results similar to those of *UAS-cac1 256B*. The remaining line was somewhat less effective, producing rescue of cac^{TS2} paralysis but not full rescue of *cac* lethal mutants to adult viability.

Rescue of temperature-sensitive paralysis. All flies used for behavioral tests were raised and maintained at room temperature (22–25°C). Paralysis at a given temperature was monitored as described previously (Dellinger et al., 2000). Briefly, groups of six flies were introduced into preheated vials and the time at which 50% of the flies were no longer standing was recorded (50% paralysis). For tests exceeding 5 min in duration, the vials were humidified at 5 min by adding water to the cotton plug sealing the vial.

Rescue of cac lethal phenotypes. Three different crosses were performed to determine the adult viability of each *cac* lethal mutant and each rescued *cac* lethal mutant relative to that of males carrying a WT X chromosome. In rescue crosses, *elav-GAL4* $l(1)L13^{20-3}/FM7i$ and *elav-GAL4* $l(1)L13^{HC129}/FM7i$ females were mated to lines homozygous for a *UAS-cac1* insertion. The extent of rescue to WT viability was measured by determining the percentage of total adult progeny represented by non-*FM7i* males (carrying the lethal mutation but rescued by transgene expression) relative to the percentage obtained from similar control crosses using +/*FM7i* females. These data were expressed as percentage viability values, which were calculated as follows: (% non-*FM7i* male progeny from the rescue cross/% non-*FM7i* male progeny from the control cross) × 100. Finally, viability of *cac* lethals in the absence of *UAS-cac1* was determined similarly in crosses of *elav-GAL4* $l(1)L13^{20-3}/FM7i$ and *elav-GAL4* $l(1)L13^{HC129}/FM7i$ females to WT (*Canton S*) males. All crosses were transferred frequently to prevent crowding of the vials.

Electrophysiology

Recordings of EPSCs from dorsal longitudinal muscle (DLM) neuromuscular synapses of the adult were obtained and analyzed as described

previously (Kawasaki et al., 1998, 2000; Kawasaki and Ordway, 2000). Recordings were performed on 2- to 4-d-old flies raised at room temperature (22–25°C).

Data analysis

Graphing and analysis of numerical data were performed in Microsoft Excel (Microsoft, Seattle, WA). All data values throughout the text and bar graphs are given as means ± SEM. Statistical analysis was performed using the two-tailed Student's *t* test; significance was assigned to comparisons for which $p \leq 0.05$.

RESULTS

A missense mutation in cac^{TS2}

To examine whether the *cac*^{TS2} mutation resides within coding sequence, the ORF of *cac* was amplified by RT-PCR and sequenced. To distinguish the induced mutation from any background polymorphisms present in the *cac*^{TS2} strain, sequence from *cac*^{TS2} was compared directly with the *cac* ORF sequence from the parent chromosome on which the *cac*^{TS2} mutation was generated (Dellinger et al., 2000) (see Materials and Methods). Putative sequence differences between *cac*^{TS2} and the parent line were re-examined using independent RNA preparations. The only nucleotide difference observed in *cac*^{TS2} (c4714t) creates a proline-to-serine missense mutation at amino acid 1385. Thus, the *cac*^{TS2} mutation maps to the second proline of a highly conserved proline pair located adjacent to the EF hand domain within the C-terminal cytoplasmic tail of the α1 subunit (Fig. 1*B*). This region of the channel is known to participate in calcium-dependent inactivation, raising the possibility that *cac*^{TS2} disrupts channel function by altering this regulatory process. By arrangement, the laboratory of Dr. Jeffrey C. Hall sequenced *cac*^{TS2} independently and identified the same missense mutation (B. Chan and J. C. Hall, Brandeis University, personal communication).

Generation of a *cac* transgene: head cDNAs reflect diversity of *cac* transcripts

As described in the introductory remarks, the *cac* gene is known to express diverse transcripts that differ with respect to mRNA splicing and editing, and possibly in their temporal and/or spatial expression patterns (Smith et al., 1996, 1998a,b; Peixoto et al., 1997). In light of this complexity, the *in vivo* expression of specific *cac*-encoded protein variants will be an important tool in examining the structural determinants of calcium-channel function. Thus, molecular constructs were generated to allow *in vivo* expression of a specific *cac* cDNA.

Transgenic expression studies required generation of *cac* cDNA clones containing a complete ORF, which have not been reported previously. Thus, RT-PCR was performed to generate *cac* cDNAs for this purpose. On the basis of previous sequence analysis (Smith et al., 1996), PCR primers were designed for RT-PCR amplification of *cac* from head RNA (see Materials and Methods). The resulting PCR products were used to generate three overlapping clones spanning the entire *cac* ORF (Fig. 2, clones A–C). As discussed in Materials and Methods, clones B and C were difficult to propagate in several bacterial strains. Multiple clones were generated and characterized for each ORF segment, revealing numerous cDNA variants that differed with respect to mRNA splicing and editing (Fig. 3). Although previously published studies of *cac* mRNA processing were performed on whole-body mRNA (Smith et al., 1996, 1998a; Peixoto et al., 1997), most of the head cDNA variants observed in the present study have been reported previously. Several exceptions are described in Figure 3 (and see Discussion). Notably, a novel site of alternative splicing was identified in the region encoding the

C-terminal cytoplasmic tail, and is referred to here as the "C-terminal variable" region (Fig. 3*A,B*). In addition to clones corresponding to previously reported *cac* cDNAs covering this region, variants containing insertions of 12 and 60 bp were observed as well. Both of these nucleotide sequences were identified as single *cac* exons in the *Drosophila* genome sequence (see Materials and Methods) and were associated with canonical splice donor and acceptor sequences at the intron–exon boundaries (Mount et al., 1992). Inclusion of the 12 or 60 bp alternative exon results in the addition of 4 or 20 aa adjacent to the IQ binding domain for calmodulin (Fig. 3*A*). The protein sequences encoded by these exons are NWKE and RKKLEHDDEHKYS-PTAVEEP, respectively. The latter amino acid sequence was used as a query in Basic Local Alignment Search Tool (BLAST) searches of nonredundant protein and translated databases (http://www.ncbi.nlm.nih.gov/BLAST); however, no clear matches outside of the *Drosophila cac* gene were found.

Clones for each of the three ORF segments were chosen to be assembled into a clone spanning the entire *cac* ORF. The specific clone selected for each segment represented the editing or splicing variant most abundant in the cDNA population and/or the most conserved with respect to vertebrate presynaptic calcium channels (Fig. 4*A*). For example, alternative exon I-IIb was included in the transgene construct because it was more abundant than I-IIa among head cDNAs and includes protein binding motifs strongly conserved with those of mammalian presynaptic calcium channels (Smith et al., 1996) (see Discussion). In addition, previous work suggests that exon I-IIa may be eye-specific (Smith et al., 1998b).

Transformation rescue of temperature-sensitive behavioral and synaptic phenotypes in cac^{TS2}

The clone including the entire *cac* ORF was shuttled into the pUAST transformation vector to produce the transgene referred to as *UAS-cac1*. The pUAST vector contains multiple binding sites for the yeast transcription factor GAL4; thus, controlled expression can be achieved using available drivers expressing GAL4 in different temporal and spatial expression patterns (Brand and Perrimon, 1993). Transgenic flies were generated to examine whether expression of the *UAS-cac1* transgene could rescue the *cac*^{TS2} paralytic phenotype. In light of previous work in embryos indicating that *cac* is expressed in the nervous system (Smith et al., 1996), as well as our recent studies showing a presynaptic role for *cac* at adult neuromuscular synapses (Kawasaki et al., 2000), we chose to drive expression specifically in the nervous system. A *GAL4* enhancer trap element in the pan-neurally expressed *elav* gene (Robinow and White, 1988; Lin and Goodman, 1994) was combined with a *UAS-cac1* transgene in a *cac*^{TS2} mutant background. Neural expression of *UAS-cac1* produced striking rescue of *cac*^{TS2} paralysis at 38°C (Fig. 4*B*), increasing the time for paralysis from 0.27 ± 0.01 min (*n* = 5) in *cac*^{TS2} alone to 39.19 ± 2.16 min (*n* = 6) in the presence of the transgene. Neither the *elav-GAL4* driver nor the *UAS-cac1* transgene alone produced rescue, as expected from the requirement for both elements to drive expression.

To examine rescue of *cac*^{TS2} in more detail, voltage-clamp analysis of synaptic currents at DLM neuromuscular synapses was performed to determine whether the synaptic phenotype of *cac*^{TS2} was also rescued. As described previously (Kawasaki et al., 2000), *cac*^{TS2} exhibited a WT synaptic current at 20°C and a marked reduction in synaptic current amplitude with respect to WT at 36°C (Fig. 5*A*). In contrast, *cac*^{TS2}*Rescue* flies carrying the

A Overview

B Alternative Exons

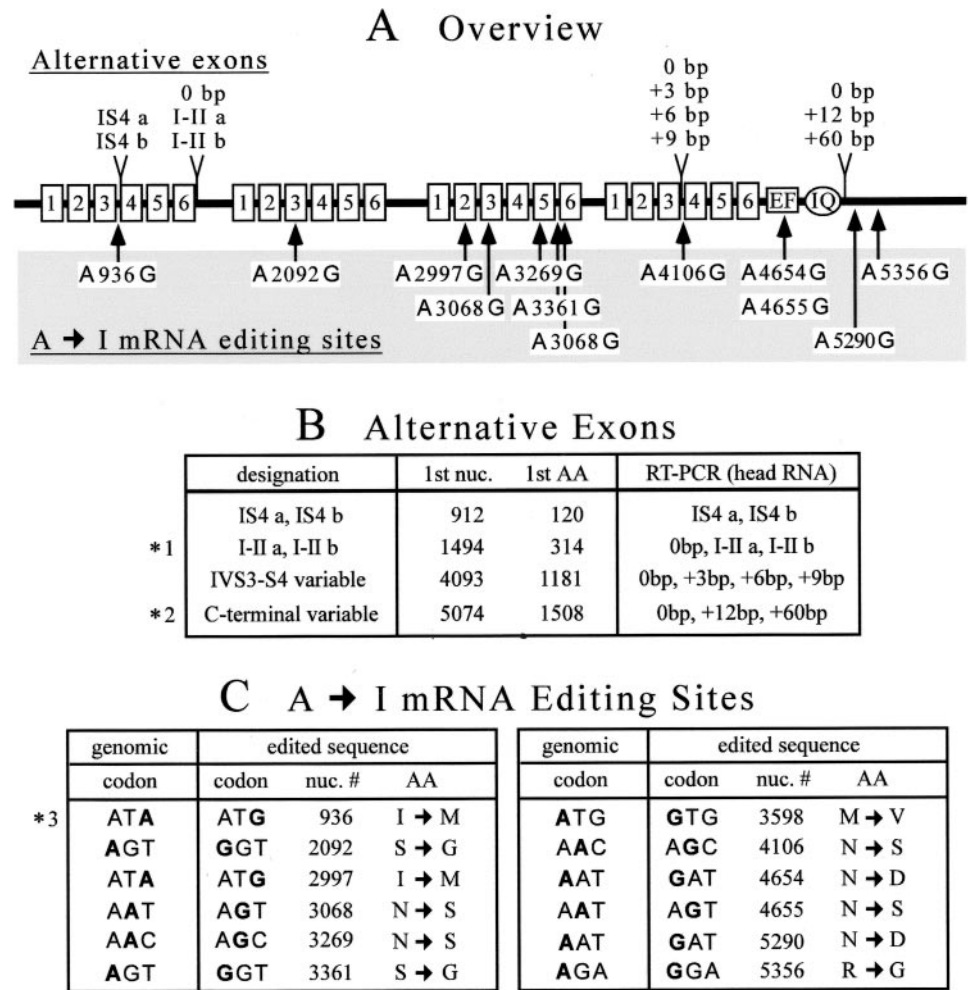|	designation	1st nuc.	1st AA	RT-PCR (head RNA)
	IS4 a, IS4 b	912	120	IS4 a, IS4 b
*1	I-II a, I-II b	1494	314	0bp, I-II a, I-II b
	IVS3-S4 variable	4093	1181	0bp, +3bp, +6bp, +9bp
*2	C-terminal variable	5074	1508	0bp, +12bp, +60bp

Figure 3. Diversity of *cac* head mRNA transcripts. Nucleotide and amino acid positions correspond to those of the *cac* cDNA sequence (U55776). Most variants were identified previously in the analysis of whole-body mRNA, with the following exceptions: *1, The variant lacking both I-IIa and I-IIb. *2, The C-terminal variable alternative exons. *3, Editing of nucleotide 936; however, this has been documented by others in recent analysis of whole body mRNA (see Discussion).

C A → I mRNA Editing Sites

genomic	edited sequence		
codon	codon	nuc. #	AA
ATA	ATG	936	I → M
AGT	GGT	2092	S → G
ATA	ATG	2997	I → M
AAT	AGT	3068	N → S
AAC	AGC	3269	N → S
AGT	GGT	3361	S → G

(*3 marks the first row)

genomic	edited sequence		
codon	codon	nuc. #	AA
ATG	GTG	3598	M → V
AAC	AGC	4106	N → S
AAT	GAT	4654	N → D
AAT	AGT	4655	N → S
AAT	GAT	5290	N → D
AGA	GGA	5356	R → G

elav-GAL4 driver and *UAS-cac1* transgene exhibited marked rescue of this synaptic phenotype (Figs. 5*A,B*). With respect to WT, synaptic current amplitudes in cac^{TS2} and $cac^{TS2}Rescue$ flies were 38.1 ± 2.6% (*n* = 4) and 80.2 ± 5.6% (*n* = 4), respectively. The above results confirm the presynaptic nature of the cac^{TS2} phenotype (Kawasaki et al., 2000) and show that the specific α1 subunit variant expressed from the *UAS-cac1* transgene can support fast, calcium-triggered neurotransmitter release (see Discussion).

Transformation rescue of *cac* lethal alleles

In addition to cac^{TS2} and other viable *cac* mutations, lethal alleles of *cac* have also been described. The lethal phase has been determined for several *cac* lethal mutants, which exhibit lethality during late embryogenesis just before hatching (Perrimon et al., 1989; Smith et al., 1998b; our unpublished observations). This late embryonic lethal stage is consistent with analysis of *cac* expression during development, which increases dramatically during late embryogenesis (Smith et al., 1996); the similar lethal phenotypes of several mutants suggest that late embryonic lethality may reflect a complete loss of zygotic *cac* function. To further explore the molecular determinants of calcium-channel function, as well as the importance of calcium-channel diversity resulting from mRNA processing, we subsequently examined whether the specific α1 subunit expressed from the *UAS-cac1* transgene could rescue *cac* lethal mutants.

Rescue of two different *cac* lethals, $l(1)L13^{20-3}$ and $l(1)L13^{HC129}$, was attempted by driving neural expression of *UAS-cac1* using the *elav-GAL4* driver. This resulted in striking rescue of the embryonic lethal phenotype in both mutants, producing viability similar to that of WT controls (Fig. 6). Routine observation of these rescued adults suggested that their motor behavior and activity were similar to WT, with the exception of wing function (see Discussion), and that they were fertile. These results indicate that neural expression of *cac* is sufficient to fulfill the essential functions of this gene. Furthermore, the specific α1 subunit expressed from *UAS-cac1* appears to provide sufficient *cac*-encoded calcium-channel activity to support robust neural function. The strong rescue of *cac* lethal mutants is surprising given that the transgene was not expressed under the control of the native *cac* promoter and that only a single variant of many possible *cac*-encoded α1 subunits was expressed.

DISCUSSION

The present study takes further advantage of the *Drosophila* genetic model system to investigate the molecular determinants of synaptic function. The findings reported here identify a missense mutation producing the TS paralytic and synaptic phenotypes in cac^{TS2}, and show that *cac* lethal and TS mutants can be rescued by neural expression of a specific *cac* cDNA. Together, these results further define the structural basis of presynaptic calcium-channel function *in vivo*.
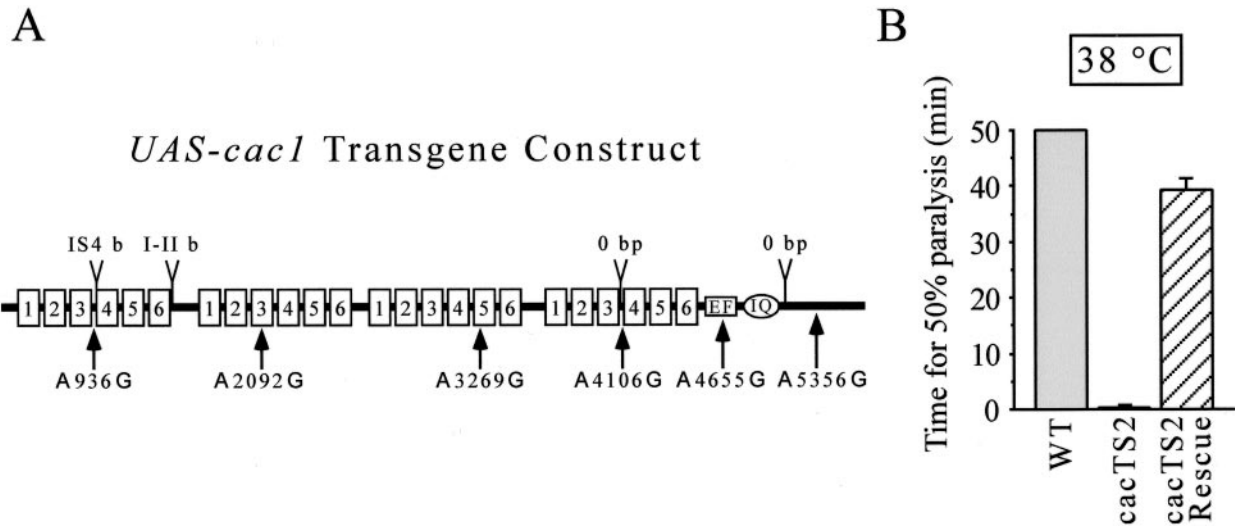
Figure 4.   Neural expression of a specific WT *cac*-encoded α1 subunit rescues TS paralysis of *cac^{TS2}*. *A*, Alternative exons and edited sequences included in the *UAS-cac1* transgene. *B*, Rescue of *cac^{TS2}* paralytic behavior. Time for 50% paralysis was measured in WT, *cac^{TS2}*, and *elav-GAL4 cac^{TS2};;UAS-cac1/+* (*cacTS2 Rescue*) at 38°C. Data and *n* values are provided in Results. Behavioral tests in WT animals were truncated after 50 min. The time for 50% paralysis values obtained in *cac^{TS2}Rescue* experiments was significantly different from that obtained for *cac^{TS2}* alone ($p \leq 0.05$).

The molecular lesion in *cac^{TS2}*

A calcium-dependent regulatory domain within the C-terminal cytoplasmic tail of the α1 subunit includes a conserved calmodulin binding site (Fig. 1*A*, *IQ*) and a conserved EF hand calcium-binding domain. Together, the IQ and EF hand motifs are thought to mediate a form of channel inactivation that is dependent on calcium influx through the open channel (Peterson et al., 2000; DeMaria et al., 2001). The *cac^{TS2}* mutation (P1385S) substitutes serine for the second proline of a highly conserved proline pair adjacent to the EF hand (Fig. 1*B*). Proline pairs are generally excluded from classical secondary structures, such as α helices and β strands, and instead are found in flexible loop and hinge regions (MacArthur and Thornton, 1991; Branden and Tooze, 1999). In light of recent models of calcium-dependent channel inactivation involving structural rearrangements of this regulatory domain (DeMaria et al., 2001; Erickson et al., 2001), it is possible that proline 1385 participates in folding transitions associated with calcium-dependent inactivation. In *cac^{TS2}*, enhanced steady-state channel inactivation at elevated temperatures might account for reduced neurotransmitter release. Additional investigation of this issue will await direct analysis of WT and *cac^{TS2}* channel behavior at permissive and restrictive temperatures.

Alternative splicing of cac head mRNA

The present study does not include a systematic analysis of alternative splicing; however, our survey of *cac* transcripts in head mRNA led to a few notable observations. One was the appearance of a novel site of alternative splicing producing variation within the C-terminal cytoplasmic tail of the α1 subunit (Fig. 3*B*). cDNA variants including either a 12 or 60 bp alternative exon at this position, as well as those lacking either exon, were observed. The close proximity of this C-terminal variable region to the IQ binding domain for calmodulin raises the possibility that the resulting channel variants may differ in their regulation by calmodulin. Given that the *UAS-cac1* transgene did not include either the 12 or 60 bp exon, these sequences are not essential for rapid neurotransmitter release.

Another novel splice variant lacked both the I-IIa and I-IIb

exons. Alternative exon I-IIb includes binding motifs for calcium-channel β subunits and heterotrimeric G-protein subunits that are highly conserved with respect to vertebrate presynaptic calcium-channel α1 subunits (Smith et al., 1998b). In contrast, exon I-IIa is less conserved and lacks a primary G-protein binding motif, QQxxRxLxGY. Thus, variation in these exons, including their absence in certain α1 subunit variants, creates the potential for differential regulation of distinct α1 subunits (Smith et al., 1998b). The conserved I-IIb alternative exon is present in the *UAS-cac1* transgene.

A final consideration is the role of alternative exons that produce variation within the IVS3–IVS4 extracellular loop. In mammalian synaptic calcium-channel α1 subunits, inclusion of NP (α1A) or ET (α1B) sequences within this loop has been reported to alter channel activation, inactivation, and pharmacology (Lin et al., 1997, 1999; Bourinet et al., 1999; Hans et al., 1999; Krovetz et al., 2000). Similar variation is generated by alternative splicing of the *cac* transcript, producing a one to three amino acid insertion at the same position. Interestingly, most *cac* mRNA variants in *Drosophila* heads lack any alternative exon in the sequence encoding the IVS3–IVS4 loop, perhaps consistent with work indicating that the analogous alternative exons in mouse α1A and α1B are enriched in the peripheral nervous system (Lin et al., 1999). It remains an open question whether variation in the IVS3–IVS4 loop of the *cac*-encoded α1 subunit serves a conserved functional role. However, the *UAS-cac1* transgene lacks alternative exons at this site, suggesting that α1 subunit sequences encoded by these exons are not critical for calcium-channel function in neurotransmitter release.

A-to-I mRNA editing

As in the case of alternative splicing, previous studies of *cac* mRNA editing have been performed using whole-fly RNA preparations. In the present study, only a qualitative survey of editing in head RNA was conducted; however, all of the reported sites were observed. In addition, sequence data were consistent with a novel editing event at nucleotide 936 (Fig. 3). On the basis of genomic and whole-fly cDNA sequence comparisons performed
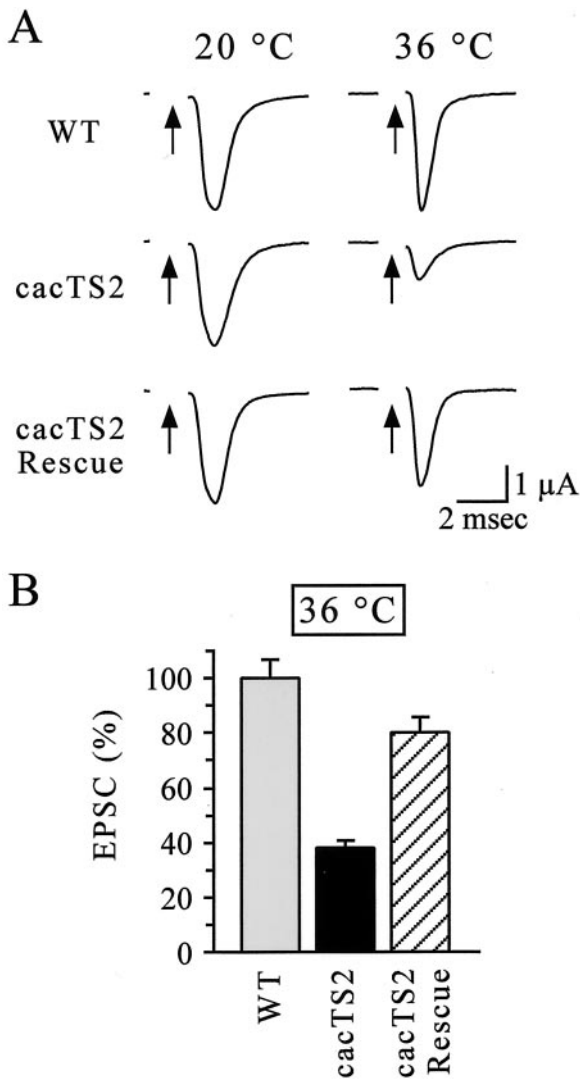
Figure 6. Rescue of *cac* lethal mutants. Three different crosses were performed to determine the adult viability of each *cac* lethal mutant and rescued *cac* lethal mutant relative to males carrying a WT X chromosome (percentage viability; see Materials and Methods). +;;*UAS-cac1*/+ males (*Control*) served as a reference for WT viability. In the absence of a *UAS-cac1* transgene, *elav-GAL4 l(1)L13²⁰⁻³* (*L13 20-3*) and *elav-GAL4 l(1)L13^{HC129}* (*L13 HC129*) males were never observed, indicating uniform lethality. In contrast, males of the genotypes *elav-GAL4 l(1)L13²⁰⁻³*;;*UAS-cac1*/+ (*L13 20-3 Rescue*) and *elav-GAL4 l(1)L13^{HC129}*;;*UAS-cac1*/+ (*L13 HC129 Rescue*) were clearly rescued and exhibited viability similar to that of WT controls. The mean percentage viability value obtained from five independent rescue experiments for each genotype ($n = 5$) is indicated. Viability of *L13 20-3 Rescue* and *L13 HC129 Rescue* males was not significantly different from that of control males ($p > 0.05$).

Rescue of *cac* mutants by neural expression of the *UAS-cac1* transgene

As discussed above, neural expression of the *UAS-cac1* transgene produced striking rescue of both TS paralytic and embryonic lethal *cac* mutants. Synaptic current recordings demonstrate that the specific $\alpha 1$ subunit variant encoded by the transgene can support fast neurotransmitter release at neuromuscular synapses and confirm previous findings demonstrating a presynaptic role for the *cac* gene product (Kawasaki et al., 2000). Because *cac* encodes the only *Drosophila* homolog of vertebrate presynaptic calcium-channel $\alpha 1$ subunits and is expressed broadly in the embryonic CNS (Smith et al., 1996), it is likely that the same *UAS-cac1* transgene product can support robust synaptic transmission in the CNS as well.

One surprising finding related to rescue of *cac* lethals was an apparent defect in the wing function of rescued adult flies. No formal behavioral analysis was performed; however, it was clear from routine observation that rescued flies were flightless and did not beat their wings under usual flight conditions. No gross morphological abnormalities were apparent in dissected preparations of *cac* lethal mutants rescued to the adult stage; however, DLM neuromuscular synapses in these preparations produced only small synaptic currents ranging from 0 to 40% of WT amplitude (our unpublished results). The reason for the observed defect in wing function remains unclear. Rescue of the *cac^{TS2}* synaptic phenotype confirms that the pan-neural *elav-GAL4* driver induces expression in the motor neurons innervating the

Figure 5. Rescue of the TS synaptic phenotype in *cac^{TS2}*. *A*, Representative synaptic current recordings from DLM neuromuscular synapses of WT, *cac^{TS2}*, and *elav-GAL4 cac^{TS2}*;;*UAS-cac1*/+ (*cacTS2 Rescue*). *Arrows* indicate stimulation of the cut motor axon. Stimulation artifacts were removed for clarity. *B*, Mean EPSC amplitudes relative to WT at 36°C. Data and *n* values are provided in Resutls. The synaptic current in *cac^{TS2}Rescue* experiments was significantly different from *cac^{TS2}* alone ($p \leq 0.05$). Comparison of the synaptic current amplitudes in *cac^{TS2}Rescue* with those of WT resulted in a *p* value of 0.073.

by others, position 936 has been confirmed as an mRNA editing site (Chan and Hall, personal communication). A subset of edited sites was present in the *UAS-cac1* transgene. Although it is possible that the mRNA produced by this transgene is edited, we consider this unlikely because editing of mRNAs, including *cac*, may require intronic sequences (Smith et al., 1998a). Thus, edited sequences absent from the transgene are probably not required for fast, calcium-triggered neurotransmitter release. With respect to the general role of editing in *Drosophila*, recent studies of a mutant thought to lack A-to-I mRNA editing of all transcripts revealed a moderate phenotype (Palladino et al., 2000). Although this mutant exhibited behavioral phenotypes and progressive deterioration of the adult nervous system, A-to-I mRNA editing in general does not appear to be essential for basic neural development or function.
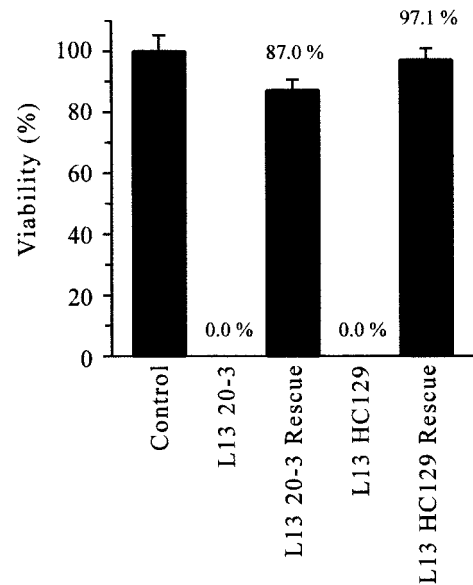
DLMs. However, proper development of these connections may require precise temporal and spatial control of *cac* expression, or perhaps a specific *cac*-encoded α1 subunit variant, that is not reproduced by expression of the *UAS-cac1* transgene under the control of *elav-GAL4*. Perhaps coincidentally, *cac* mutants were first identified in a phenotypic screen for alterations in the precise wing-beat patterns comprising the male courtship song (von Schilcher, 1976, 1977; Smith et al., 1998b). In combination with viable *cac* mutants exhibiting song phenotypes, the availability of *cac* transgenes that may be driven in different temporal and spatial expression patterns may provide new insights into the cellular and molecular basis of the courtship song.

Absence of a conserved SYNPRINT domain within the calcium-channel α1 subunit does not prevent rapid coupling of calcium influx to neurotransmitter release

Previous work on the *Drosophila cac* gene indicated that the *Drosophila* genome contains no sequences related to characterized calcium-channel synaptic protein binding domains (Kawasaki et al., 2000; Littleton and Ganetzky, 2000). The present study extends this observation by demonstrating directly that a specific calcium-channel α1 subunit lacking these sequences functions in fast, calcium-triggered neurotransmitter release. These findings indicate either that the *cac*-encoded α1 subunit contains an analogous but distinct synaptic protein binding domain or that such domains do not play a critical role in synaptic transmission. Recent work in another system has shown that syntaxin-mediated enhancement of slow calcium-channel inactivation does not require the SYNPRINT domain, although this regulation was more effective on SYNPRINT-containing channels (Bezprozvanny et al., 2000). Together, these results suggest that multiple biochemical and functional interactions between calcium-channel α1 subunits and synaptic proteins may contribute to regulation of neurotransmitter release.

The present study further defines the determinants of synaptic calcium-channel function in *Drosophila* through molecular analysis of *cac^{TS2}*, as well as rescue of *cac* mutants by neural expression of a specific *cac*-encoded α1 subunit. These findings also serve as the basis for additional analysis in this model system, including direct studies of TS calcium-channel function produced by the *cac^{TS2}* mutation, analysis of second-site *cac* mutations that either enhance or suppress the *cac^{TS2}* phenotype (Brooks et al., 2002), and a variety of investigations that require controlled expression of specific *cac*-encoded α1 subunits *in vivo*.

REFERENCES

Bezprozvanny I, Scheller RH, Tsien RW (1995) Functional impact of syntaxin on gating of N-type and Q-type calcium channels. Nature 378:623–626.

Bezprozvanny I, Zhong P, Scheller RH, Tsien RW (2000) Molecular determinants of the functional interaction between syntaxin and N-type Ca²⁺ channel gating. Proc Natl Acad Sci USA 97:13943–13948.

Bourinet E, Soong TW, Sutton K, Slaymaker S, Mathews E, Monteil A, Zamponi GW, Nargeot J, Snutch TP (1999) Splicing of α1A subunit gene generates phenotypic variants of P- and Q-type calcium channels. Nat Neurosci 2:407–415.

Brand AH, Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118:401–415.

Branden C, Tooze J (1999) Introduction to protein structure, Ed 2. New York: Garland.

Brooks IM, Felling R, Godeny MD, Ordway RW (2002) Genetic modifiers of a temperature-sensitive paralytic calcium channel mutant of *Drosophila*: genetic, molecular and phenotypic characterization. Paper presented at the 43rd Annual *Drosophila* Research Conference, San Diego.

Catterall WA (1998) Structure and function of neuronal Ca²⁺ channels and their role in neurotransmitter release. Cell Calcium 24:307–323.

Colecraft HM, Brody DL, Yue DT (2001) G-protein inhibition of N- and P/Q-type calcium channels: distinctive elementary mechanisms and their functional impact. J Neurosci 21:1137–1147.

Degtiar VE, Scheller RH, Tsien RW (2000) Syntaxin modulation of slow inactivation of N-type calcium channels. J Neurosci 20:4355–4367.

Dellinger BB, Felling R, Ordway RW (2000) Genetic modifiers of the *Drosophila* NSF mutant, *comatose*, include a temperature-sensitive paralytic allele of the calcium channel α1 subunit gene, *cacophony*. Genetics 155:203–211.

DeMaria CD, Soong TW, Alseikhan BA, Alvania RS, Yue DT (2001) Calmodulin bifurcates the local Ca²⁺ signal that modulates P/Q-type Ca²⁺ channels. Nature 411:484–489.

De Waard M, Liu H, Walker D, Scott VES, Gurnett AA, Campbell KP (1997) Direct binding of G-protein βγ complex to voltage-dependent calcium channels. Nature 385:446–450.

Dove LS, Abbott LC, Griffith WH (1998) Whole-cell and single-channel analysis of P-type calcium currents in cerebellar Purkinje cells of leaner mutant mice. J Neurosci 18:7687–7699.

Dunlap K (1997) Integration hot-spot gets hotter. Nature 385:394–397.

Erickson MG, Alseikhan BA, Peterson BZ, Yue DT (2001) Preassociation of calmodulin with voltage-gated Ca²⁺ channels revealed by FRET in single living cells. Neuron 31:973–985.

Gielow ML, Gu G-G, Singh S (1995) Resolution and pharmacological analysis of the voltage-dependent calcium channels of *Drosophila* larval muscles. J Neurosci 15:6085–6093.

Hans M, Urrutia A, Deal C, Brust PF, Stauderman K, Ellis SB, Harpold MM, Johnson EC, Williams ME (1999) Structural elements in domain IV that influence biophysical and pharmacological properties of human α1A-containing high-voltage-activated calcium channels. Biophys J 76:1384–1400.

Herlitze S, Zhong H, Scheuer T, Catterall WA (2001) Allosteric modulation of Ca²⁺ channels by G proteins, voltage-dependent facilitation, protein kinase C, and Ca_vβ subunits. Proc Natl Acad Sci USA 98:4699–4704.

Higuchi M, Maas S, Single FN, Hartner J, Rozov A, Burnashev N, Feldmeyer D, Sprengel R, Seeburg PH (2000) Point mutation in an AMPA receptor gene rescues lethality in mice deficient in the RNA-editing enzyme ADAR2. Nature 406:78–81.

Ikeda SR, Dunlap K (1999) Voltage-dependent modulation of N-type calcium channels: role of G protein subunits. Adv Second Messenger Phosphoprotein Res 33:131–151.

Ino M, Yoshinaga T, Wakamori M, Miyamoto N, Takahashi E, Sonoda J, Kagaya T, Oki T, Nagasu T, Nishizawa Y, Tanaka I, Imoto K, Aizawa S, Koch S, Schwartz A, Niidome T, Sawada K, Mori Y (2001) Functional disorders of the sympathetic nervous system in mice lacking the α1B subunit (Ca_v 2.2) of N-type calcium channels. Proc Natl Acad Sci USA 98:5323–5328.

Jarvis SE, Zamponi GW (2001) Distinct molecular determinants govern syntaxin 1A-mediated inactivation and G-protein inhibition of N-type calcium channels. J Neurosci 21:2939–2948.

Jun K, Piedras-Renteria ES, Smith SM, Wheeler DB, Lee SB, Lee TG, Chin H, Adams ME, Scheller RH, Tsien RW, Shin H-S (1999) Ablation of P/Q-type Ca²⁺ channel currents, altered synaptic transmission, and progressive ataxia in mice lacking the α1A-subunit. Proc Natl Acad Sci USA 96:15245–15250.

Karess RE, Rubin GM (1984) Analysis of P transposable element functions in *Drosophila*. Cell 38:135–146.

Kawasaki F, Ordway RW (2000) Fast synaptic fatigue in *shibire* mutants reveals a rapid requirement for dynamin in synaptic vesicle membrane trafficking. Nat Neurosci 3:859–860.

Kawasaki F, Mattiuz AM, Ordway RW (1998) Synaptic physiology and ultrastructure in comatose mutants define an *in vivo* role for NSF in neurotransmitter release. J Neurosci 18:10241–10249.

Kawasaki F, Felling R, Ordway RW (2000) A temperature-sensitive paralytic mutant defines a primary synaptic calcium channel in *Drosophila*. J Neurosci 20:4885–4889.

Krovetz HS, Helton TD, Crews AL, Horne WA (2000) C-terminal alternative splicing changes the gating properties of a human spinal cord calcium channel α1A subunit. J Neurosci 20:7564–7570.

Lee A, Wong ST, Gallagher D, Li B, Storm DR, Scheuer T, Catterall WA (1999) Ca²⁺/calmodulin binds to and modulates P/Q-type calcium channels. Nature 399:155–159.

Lin DM, Goodman CS (1994) Ectopic and increased expression of Fasciclin II alters motoneuron growth cone guidance. Neuron 13:507–523.

Lin Z, Haus S, Edgerton J, Lipscombe D (1997) Identification of functionally distinct isoforms of the N-type Ca²⁺ channel in rat sympathetic ganglia and brain. Neuron 18:153–166.

Lin Z, Lin Y, Schorge S, Qian Pan J, Beierlein M, Lipscombe D (1999) Alternative splicing of a short cassette exon in α_1B generates functionally distinct N-type calcium channels in central and peripheral neurons. J Neurosci 19:5322–5331.

Littleton TJ, Ganetzky B (2000) Ion channels and synaptic organization: analysis of the *Drosophila* genome. Neuron 26:36–43.

Lorenzon NM, Lutz CM, Frankel WN, Beam KG (1998) Altered cal-

cium channel currents in Purkinje cells of the neurological mutant mouse leaner. J Neurosci 18:4482–4489.

Lü Q, AtKisson MS, Jarvis SE, Feng ZP, Zamponi GW, Dunlap K (2001) Syntaxin 1A supports voltage-dependent inhibition of α1B Ca^{2+} channels by $G\beta\gamma$ in chick sensory neurons. J Neurosci 21:2949–2957.

MacArthur MW, Thornton JM (1991) Influence of proline residues on protein conformation. J Mol Biol 218:397–412.

Mirotznik RR, Zheng X, Stanley EF (2000) G-protein types involved in calcium channel inhibition at a presynaptic nerve terminal. J Neurosci 20:7614–7621.

Mochida S, Sheng Z-H, Carl B, Kobayashi H, Catterall WA (1996) Inhibition of neurotransmission by peptides containing the synaptic protein interaction site of N-type Ca^{2+} channels. Neuron 17:781–788.

Mount SM, Burks C, Hertz G, Stormo GD, White O, Fields C (1992) Splicing signals in *Drosophila*: intron size, information content, and consensus sequences. Nucleic Acids Res 20:4255–4262.

Palladino MJ, Keegan LP, O'Connell MA, Reenan RA (2000) A-to-I pre-mRNA editing in *Drosophila* is primarily involved in adult nervous system function and integrity. Cell 102:437–449.

Pan JQ, Lipscombe D (2000) Alternative splicing in the cytoplasmic II–III loop of the N-type Ca channel α1B subunit: functional differences are β subunit-specific. J Neurosci 20:4769–4775.

Peixoto AA, Smith LA, Hall JC (1997) Genomic organization and evolution of alternative exons in a *Drosophila* calcium channel gene. Genetics 145:1003–1013.

Perrimon N, Engstrom L, Mahowald AP (1989) Zygotic lethals with specific maternal effect phenotypes in *Drosophila melanogaster*. I. Loci on the X chromosome. Genetics 121:333–352.

Peterson BZ, Lee JS, Mulle JG, Wang Y, de Leon M, Yue DT (2000) Critical determinants of Ca^{2+}-dependent inactivation within an EF-hand motif of L-type Ca^{2+} channels. Biophys J 78:1906–1920.

Reenan RA (2001) The RNA world meets behavior: A to I pre-mRNA editing in animals. Trends Genet 17:53–56.

Ren D, Xu H, Eberl DF, Chopra M, Hall LM (1998) A mutation affecting dihydropyridine-sensitive current levels and activation kinetics in *Drosophila* muscle and mammalian heart calcium channels. J Neurosci 18:2335–2341.

Robinow S, White K (1988) The locus *elav* of *Drosophila melanogaster* is expressed in neurons at all developmental stages. Dev Biol 126:294–303.

Saegusa H, Kurihara T, Zong S, Minowa O, Kazuno A, Han W, Matsuda Y, Yamanaka H, Osanai M, Noda T, Tanabe T (2000) Altered pain responses in mice lacking α1E subunit of the voltage-dependent Ca^{2+} channel. Proc Natl Acad Sci USA 97:6132–6137.

Schafer WR, Kenyon CJ (1995) A calcium-channel homologue required for adaptation to dopamine and serotonin in *Caenorhabditis elegans*. Nature 375:73–78.

Sheng Z-H, Westenbroek RE, Catterall WA (1998) Physical link and functional coupling of presynaptic calcium channels and the synaptic vesicle docking/fusion machinery. J Bioenerg Biomembr 30:335–345.

Smith LA, Wang X, Peixoto AA, Neumann EK, Hall LM, Hall JC (1996) A *Drosophila* calcium channel α1 subunit gene maps to a genetic locus associated with behavioral and visual defects. J Neurosci 16:7868–7879.

Smith LA, Peixoto AA, Hall JC (1998a) RNA editing in the *Drosophila* Dmca1A calcium-channel α1 subunit transcript. J Neurogenet 12:227–240.

Smith LA, Peixoto AA, Kramer EM, Villella A, Hall JC (1998b) Courtship and visual defects of *cacophony* mutants reveal functional complexity of a calcium-channel α1 subunit in *Drosophila*. Genetics 149:1407–1426.

Stanley EF (1997) The calcium channel and the organization of the presynaptic transmitter release face. Trends Neurosci 20:404–409.

von Schilcher F (1976) The behavior of *cacophony*, a courtship song mutant in *Drosophila melanogaster*. Behav Biol 17:187–196.

von Schilcher F (1977) A mutation which changes courtship song in *Drosophila melanogaster*. Behav Genet 7:251–259.

Walker D, De Waard M (1998) Subunit interaction sites in voltage-dependent Ca^{2+} channels: role in channel function. Trends Neurosci 21:148–154.

Wu MN, Fergestad T, Lloyd TE, He Y, Broadie K, Bellen HJ (1999) Syntaxin 1A interacts with multiple exocytic proteins to regulate neurotransmitter release in vivo. Neuron 23:593–605.

Yang J, Tsien RW (1993) Enhancement of N- and L-type calcium channel currents by protein kinase C in frog sympathetic neurons. Neuron 10:127–136.

Zamponi GW, Bourinet E, Nelson D, Nargeot J, Snutch TP (1997) Crosstalk between G proteins and protein kinase C mediated by the calcium channel α1 subunit. Nature 385:442–446.

Zheng W, Feng G, Ren D, Eberl DF, Hannan F, Dubald M, Hall LM (1995) Cloning and characterization of a calcium channel α1 subunit from *Drosophila melanogaster* with similarity to the rat brain type D isoform. J Neurosci 15:1132–1143.

Zhong H, Yokoyama CT, Scheuer T, Catterall WA (1999) Reciprocal regulation of P/Q-type Ca^{2+} channels by SNAP-25, syntaxin and synaptotagmin. Nat Neurosci 2:939–941.