Neurochem. **52**, 521 (1989)]. After preparation, the synaptosomes were suspended in Hepes-buffered saline containing 142 mM NaCl, 2.4 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 1.2 mM K_2HPO_4, 10 mM glucose, and 10 mM Hepes, pH 7.2, with a final protein concentration of 1 mg per milliliter of suspension. The synaptosomes were loaded with radiolabeled neurotransmitter by incubation in 2 ml of 0.2 μM [^{3}H]NE (11 Ci/mmol) for 10 min at 37°C in Hepes-buffered saline. Then, 1 ml of synaptosome suspension was placed on filters (Whatman glass-microfiber filters; 0.7-μm pore size) in 10 parallel wells over a vacuum manifold, with each well being used for a single release condition. Hepes-buffered saline was the carrier solution for all agents; each wash was 2 ml and required about 1 s. Sequential washes were applied at one per minute. After seven initial washes, all washes were collected and the tritium quantitated. At the end of each experiment, the filter paper was also collected and counted. The percent of radioactivity released at each wash was calculated as a fraction of the total amount of radioactivity recovered during an experiment (after baseline had been established). This total included residual counts on the filter paper and unreleased counts in the synaptosomes (after lysis). Total release percentages from the stimulus sample plus the two following samples were normalized to the maximum amount released in a given experiment. In the experiments with Hgb, corrections for quenching were made. The L-Glu release was measured with a bioluminescence assay [V. M. Fosse *et al.*, *J. Neurochem.* **47**, 340 (1986)]. The procedure for computing the percentage of L-Glu released during each wash was analogous to that used for [^{3}H]NE release. The bioluminescence measurements for endogenous L-Glu release were performed concurrently with a Monolight (Analytical Luminescence Laboratory, San Diego, CA) 2010 luminometer on a 50-μl aliquot of each wash. Measurements were linear over the range of 10^{-7} to 10^{-6} M. After an experiment, the synaptosomes were osmotically ruptured, and the residual L-Glu remaining on the filters was measured and added to the total amount of L-Glu recovered during the washes. The percentage released was expressed as a fraction of this total.

17. The K^+ depolarization did not nonspecifically damage the synaptosomes because the NMDA-induced release remained intact after this depolarization and the depolarization-induced release was Ca^{2+} dependent.
18. For a given experiment, multiple aliquots from the same batch of synaptosomes would release a relatively constant fraction of the available transmitter (*15*). The variability originated primarily from batch to batch variation.
19. P. K. Moore, R. C. Babbedge, P. Wallace, Z. A. Gaffen, S. L. Hart, *Br. J. Pharmacol.* **108**, 296 (1993).
20. M. A. Dwyer, D. S. Bredt, S. H. Snyder, *Biochem. Biophys. Res. Commun.* **176**, 1136 (1991).
21. J. Garthwaite, *Trends Neurosci.* **14**, 60 (1991); D. S. Bredt and S. H. Snyder, *Neuron* **8**, 3 (1992). We added Hgb either in the unmodified met form or as a more purified oxyhemoglobin prepared by reduction with sodium dithionite followed by reoxidation by gassing with O_2 followed by dialysis to remove the dithionite.
22. D. S. Bredt, P. M. Hwang, S. H. Snyder, *Nature* **347**, 768 (1990); S. H. Snyder and D. S. Bredt, *Trends Pharmacol. Sci.* **12**, 125 (1991); T. M. Dawson, V. L. Dawson, S. H. Snyder, *Ann. Neurol.* **32**, 297 (1992); S. R. Vincent and B. T. Hope, *Trends Neurosci.* **15**, 108 (1992); S. R. Vincent and H. Kimura, *Neuroscience* **46**, 755 (1992); C. W. Picanco-Diniz *et al.*, *Soc. Neurosci Abstr.* **18**, 210 (1992); J. G. Valtschanoff, R. J. Weinberg, V. N. Kharazia, M. Nakane, H. H. H. W. Schmidt, *J. Comp. Neurol.* **331**, 111 (1993).
23. The effluent from each batch of synaptosomes was delivered to two separate rat aortic rings connected by surgical suture to separate Statham (Gould Instruments Division, Atlanta, GA) force transducers. The endothelium of these aortic rings was removed so that their NO would not be available as a relaxant in this system. In each experiment, the aortic rings were first contracted with 1×10^{-8} to 3×10^{-8} M U46619 (a thromboxane analog) to give a broad working range for measurable changes in tension, and the rings were constantly exposed to the U46619 for the duration of the experiment [M. J. Winn *et al.*, *J. Pharmacol. Toxicol. Methods* **28**, 49 (1992)].
24. R. F. Furchgott and J. Z. Zawadzki, *Nature* **288**, 373 (1980); F. R. Furchgott and P. M. Vanhoutte, *FASEB J.* **3**, 2007 (1989).
25. It is possible that the baseline release of the neurotransmitters combined with the pulsatile release in response to the 100 μM NMDA stimulus directly caused the vasodilatory response. To control for this possibility, we computed the amount of L-Glu and [^{3}H]NE released during the NMDA stimulus wash and the succeeding two washes and exposed the aortic rings to these concentrations over a 3-min period. Neither L-Glu (5 to 50 μM), [^{3}H]NE (1 to 20 μM), nor Ach (1 μM) caused any change in ring tension.
26. M. J. Winn, D. D. Ku, J. M. Nelson, *J. Cardiovasc. Pharmacol.* **18**, 68 (1991); J. A. Bellan, L. L. Longenecker, P. J. Kaolowitz, D. B. McNamara, *Eur. J. Pharmacol.* **234**, 273 (1993).
27. Nitric oxide gas was applied directly by first dissolving NO in helium-saturated distilled water. The resulting concentration was determined by the method of M. Feelisch and E. A. Noack [*Eur. J. Pharmacol.* **139**, 19 (1987)].
28. N. Bettache *et al.*, *Biophys. J.* **64** (suppl.), 190 (1993).
29. J. S. Stamler, D. J. Singel, J. Loscalzo, *Science* **258**, 1898 (1992).
30. J. W. Ferkany *et al.*, *Nature* **298**, 757 (1982); K. Fink *et al.*, *Eur. J. Pharmacol.* **185**, 115 (1990); J. K. T. Wang *et al.*, *J. Neurochem.* **58**, 204 (1992).
31. O. Manzoni, *Neuron* **8**, 653 (1992); Y. Izumi, D. Clifford, C. F. Zorumski, *Science* **257**, 1273 (1992); O. Manzoni and J. Bockaert, *J. Neurochem.* **61**, 368 (1993); S. A. Lipton *et al.*, *Nature* **364**, 626 (1993).
32. We thank F. Hester for luminometer measurements, J. Beckman for providing NO and help with its application and measurement, T. Sejnowski, J. Hablitz, S. Nowlan, and P. Dayan for helpful comments on an earlier draft of the manuscript, C. Stevens, M. Nusbaum, and J. Beckman for helpful discussions, J. Neville and L. A. Faulkner for word processing, K. Ramer for figure preparation, and N. Veyna for synaptosome and luminometry protocols. Supported by NIH EY05116 (M.J.F.) and NIH EY06714 (R.B.M.).

23 July 1993; accepted 4 January 1994

Paralysis and Early Death in Cysteine String Protein Mutants of *Drosophila*

Konrad E. Zinsmaier,*† Kai K. Eberle,† Erich Buchner, Niklaus Walter, Seymour Benzer

Multimeric complexes of synaptic vesicle and terminal membrane proteins are important components of the neurotransmitter release mechanism. The *csp* gene of *Drosophila* encodes proteins homologous to synaptic vesicle proteins in *Torpedo.* Monoclonal antibodies demonstrate different distributions of isoforms at distinct subsets of terminals. Deletion of the *csp* gene in *Drosophila* causes a temperature-sensitive block of synaptic transmission, followed by paralysis and premature death.

Fast, excitation-coupled neurotransmitter release is mediated by the fusion of synaptic vesicles with the nerve terminal. Proteins of both vesicles and other synaptic compartments, including presynaptic Ca^{2+} channels, participate in this process by forming a multimeric protein complex to ensure rapid and regulated release (*1*). Although the molecular mechanisms are not understood, considerable progress has been made in identifying some of the components (*2*). However, only synaptotagmin has thus far been genetically shown to be involved (*3*).

The *Drosophila melanogaster* cysteine string proteins (Dcsp's) (*4*) are membrane proteins containing an unusual cysteine motif ($C_2X_5\ C_{11}\ X_2\ C_2$; C is cysteine and X is any amino acid) and the "J" domain found in dnaJ proteins, which mediate the regulation of heat shock protein (hsp) 70 protein activity and thereby the assembly of multimeric protein complexes (*5*). A *csp* homolog of *Torpedo californica* (Tcsp) has been identified (*6*) that copurifies with synaptic vesicles (*7*). The inhibition of N-type Ca^{2+} channel activity by coinjection of *Tcsp* antisense mRNA into *Xenopus* oocytes suggests that Tcsp may be a regulatory subunit of Ca^{2+} channels (*6*). We show that, in *Drosophila*, the Dcsp proteins are localized to synaptic terminals and that deletion of the *csp* gene interferes with synaptic transmission, causing paralysis and early death.

To localize the gene product, we generated monoclonal antibodies (mAbs) from fusion protein (*8*). The DCSP-2 mAb demonstrates the presence of Dcsp protein presynaptically at larval and adult neuromuscular junctions; synaptic boutons are strongly stained (Fig. 1, A to C). In the central nervous system, synapse-rich regions of the neuropil are positive, whereas the surrounding cell bodies are negative, as exemplified

K. E. Zinsmaier and S. Benzer, California Institute of Technology, Division of Biology 156-29, Pasadena, CA 91125, USA.
K. K. Eberle and E. Buchner, Theodor-Boveri-Institut für Biowissenschaften, Am Hubland, D-97074 Würzburg, Germany.
N. Walter, Zoologisches Institut, Winterthurerstr. 190, CH-Zürich 8057, Switzerland.

*To whom correspondence should be addressed.
†These authors contributed equally to this work.

for the visual system in Fig. 1B.

The DCSP-3 mAb detects isoforms predominantly expressed in the photoreceptor terminals (Fig. 1D), but not at motor nerve terminals (Fig. 1E). The exclusive localization of Dcsp to synaptic terminals in *Drosophila* suggests an association with synaptic vesicles. Indeed, immunoprecipitation with antibody to *Drosophila* synaptotagmin (*9*) coprecipitates Dcsp's (*10*), providing evidence for a similar synaptic vesicle localization as in *Torpedo*.

We analyzed the *csp* gene (*11*), which expresses three low-abundance transcripts (Fig. 2, A and B) exclusively in neurons as demonstrated by in situ hybridization (*4*). The *csp* locus is at position 79E1-2 on chromosome 3 (*4*). Because no chromosomal rearrangements of this region were available, we used site-selected P-element mutagenesis (*12*) to target the gene. Twenty-four thousand P-element insertion lines were screened and two, csp^{P1} and csp^{P2}, in close proximity to the *csp* transcription unit, were isolated (Fig. 2A). These were used to induce gene deletions (*13*). Genetic analysis identified two complementation groups (Fig. 3A). Deletion group I mutants exhibit a semilethal embryonic phenotype; adult escapers show early death (Fig. 3B) and the temperature-sensitive (ts) paralytic behavior described below. Deletion group II mutants show a strong embryonic lethal phenotype; they apparently correspond to a neighboring gene 970 base pairs (bp) upstream of *csp* (Fig. 2, A and B).

Two group I deletions were molecularly analyzed in detail. The allele csp^{R1} deletes the entire locus, representing a null mutation of the *csp* gene (Fig. 2A). The second deletion, csp^{X1}, removes the *csp* promoter and the first exon of the gene (Fig. 2A). The molecular analysis showed that only deletions of com-

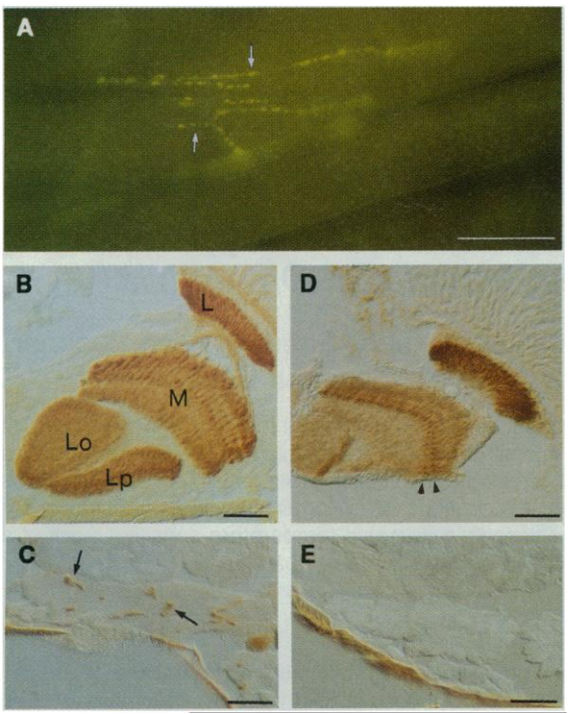
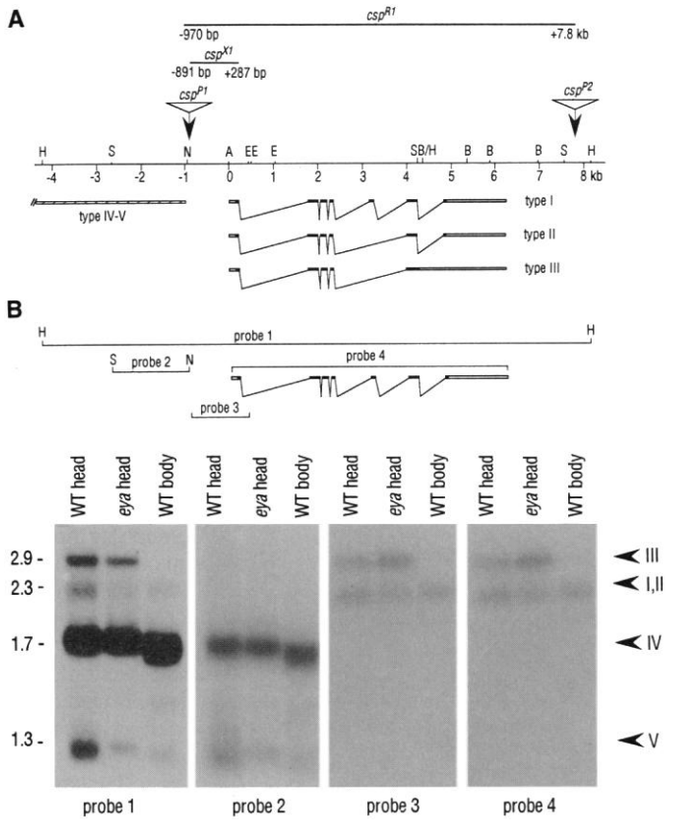


Fig. 1 (left). Differential expression of Dcsp's at distinct synaptic terminals. The DCSP-2 mAb, which identifies all the cysteine string proteins, detects expression in a wide range of terminals, including the larval neuromuscular junctions (**A**), the adult visual system (**B**), and adult thoracic neuromuscular junctions (**C**). Note the strong staining of neuropils of the optic ganglia (L, M, Lo, Lp) (B), whereas the surrounding cell bodies are unstained. Fiber tracts containing axons but not synapses appear to contain little Dcsp protein. At motor nerve terminals, Dcsp is strongly expressed at synaptic boutons (arrows), but the axons of the motor neurons are not stained (A and C). Specific Dcsp proteins are expressed at distinct nerve terminals, as indicated by DCSP-3 mAb (**D** and **E**), which identifies a subset of Dcsp proteins expressed predominantly in the terminals of photoreceptor cells R1-6, which innervate the lamina (L), and the R7-8 (arrowheads) terminating in the medulla (M), but not in the presynaptic endings of motor neurons (E). (Note: the cuticle is naturally brown.) Immunohistochemistry was as described (*19*). Abbreviations: Lo, lobula; Lp, lobula plate. Bar, 50 μm. **Fig. 2 (right).** Molecular analysis of the *csp* gene. (**A**) The restriction map of the *csp* locus is shown within the region covered by the λ-fix phage 9-8 (*8*). Position 0 indicates the starting point of *csp* transcription. Parts of this region were sequenced (*8*) and the exon-intron structure determined. Three alternatively spliced *csp* cDNAs (types I to III) are shown. Noncoding regions are indicated by open boxes; closed boxes indicate coding sequences. The shaded box corresponds to a neighboring transcription unit that expresses two RNA transcripts, types IV and V, which do not extend beyond the csp^{P1} P insertion (compare with Fig. 2B). The map



positions of the two P-element insertions csp^{P1} (−891) and csp^{P2} (+7.8) targeting the *csp* gene are indicated. The extent of the chromosomal rearrangements (bars above the restriction map) of csp^{R1} and csp^{X1} were determined by sequence analysis of their breakpoints; their map positions are indicated. The deletion csp^{R1} cuts out the entire *csp* transcription unit. Csp^{X1} is a small deletion of 1178 bp that uncovers the first *csp* exon and the promoter region. (**B**) The *csp* genomic region (A) was analyzed for transcription activity by Northern (RNA) blotting (*20*), and five different transcripts were identified (probe 1: Hind III–Hind III, 12.3 kb). Transcript types I to III are detected with the type I cDNA (probe 4); they belong to the *csp* transcription unit. Transcripts types I and II migrate on Northern blots at 2.4 kb as a double band due (*11*) to their small size difference of only 60 bp; type III migrates at 2.9 kb and is head-specific. The highly abundant transcript types IV and V (probe 2: Sal I–Nde I, 1.7 kb; shaded box in Fig. 2A) are detected by genomic fragments from positions −4000 to −791. The corresponding gene was identified as an RNA helicase and a member of the DEAD box protein family (*10*). A genomic fragment (probe 3: polymerase chain reaction fragment from position −891 to +482) representing the deleted region of csp^{X1} detects only the low-abundance *csp* transcripts, but not the highly expressed neighboring transcript types VI and V. Molecular size markers are indicated on the left (in kilodaltons).

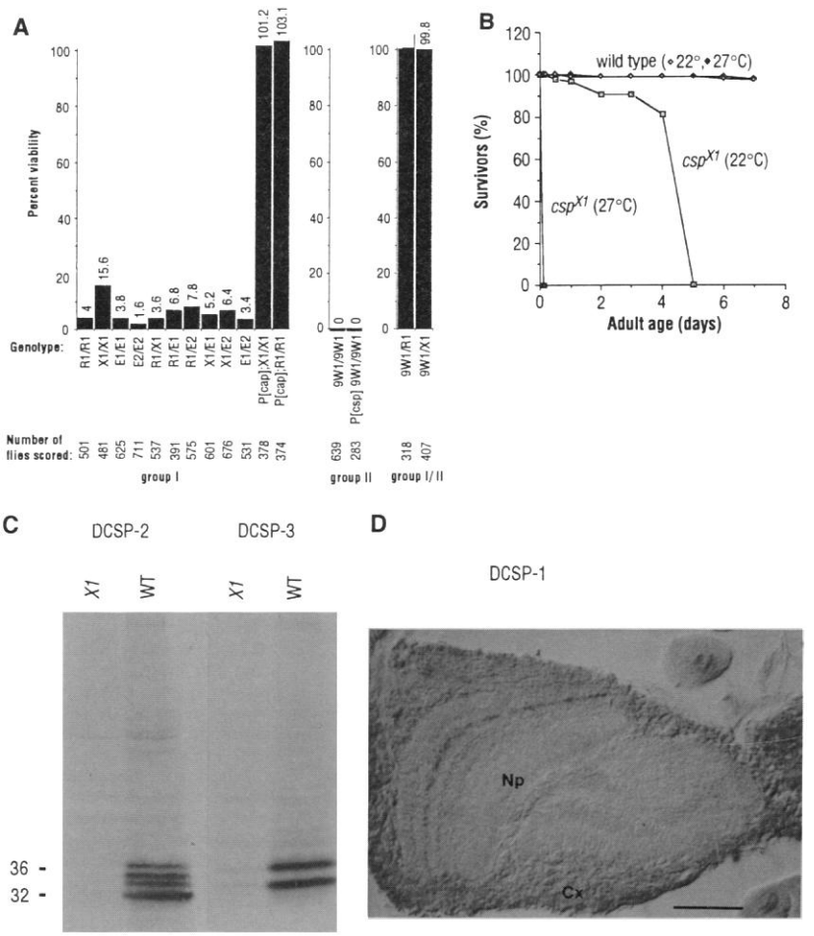


Fig. 3. Genetic analysis of *csp* mutants and protein expression of csp^{X1}. (**A**) Genetic analysis of *csp* mutants. Complementation analysis of group I (left) with the molecularly analyzed deletions csp^{X1} and csp^{R1} and two further EMS alleles csp^{E1} and csp^{E2}. All combinations are semilethal during development, allowing only a few flies to survive to adulthood. The P rescue element P[w^+, *csp*] (*14*) completely rescues the developmental lethality of csp^{X1} and csp^{R1}, but not the strong recessive lethal phenotype of the complementation group II (middle). On the right side of the graph, complementation is shown between *csp* alleles and one member of the second complementation group; they complement each other fully. Flies were counted within 24 hours of eclosion. (**B**) Adult mutant *csp* flies die prematurely after 4 to 5 days at 22°C, as exemplified by csp^{X1}. At higher temperature (29°C), flies become paralyzed and die within an hour; wild-type flies are not affected at either temperature, and their life-span is over 35 days. All flies were raised at 18°C, newly emerged flies were transferred to the indicated temperature, and dead flies were counted every 12 hours. (**C**) Immunostaining of protein immunoblots or immunohistochemical staining with all antibodies shows almost complete absence of Dcsp proteins in csp^{X1} (*21*). Whether the residual staining represents nonspecific background or faint Dcsp expression is unclear, but the genetics predict a hypomorphic allele. On immunoblots, DCSP-2 mAb (left) detects Dcsp proteins in wild-type flies ranging in size from 32 to 36 kD; these are highly reduced in csp^{X1}. DCSP-3 mAb specifically detects two of the Dcsp isoforms; they are almost absent in the mutant. Molecular size markers are indicated on the left (in kilodaltons). (**D**) Immunohistochemical test of DCSP-1 mAb [former ab49 (*4*] stain on the optic lobes in the mutant csp^{X1} shows almost no Dcsp expression. Note that the neuropil is much lighter than the cortical cells (compare to wild type in Fig. 1). Cx, cortex; Np, neuropil. Bar, 50 μm.

plementation group I affect exclusively the *csp* transcription unit. To test whether the ts paralytic behavior of these mutants indeed corresponds to the *csp* gene, we used a genomic DNA fragment to rescue the mutant phenotype (*14*). Both the lethality and the early adult death of csp^{R1} and csp^{X1} were rescued by the construct P[w^+, csp^+] (Fig. 3B), which includes the entire *csp* transcription unit and parts of the neighboring transcription unit (Fig. 2). Mutants of the second complementation group were not rescued (Fig. 3A). This confirms that the ts lethality and paralytic behavior were caused by mutations of the *csp* gene.

The phenotypes of the analyzed *csp* deletion mutants are surprising. The mutant with a complete deletion of the *csp* gene, csp^{R1}, is a recessive, ts embryonic semilethal; 4% escape to adulthood at 25°C, but none escapes at 29°C. Lethality is first observed in 18- to 24-hour-old embryos, which seem to develop properly; antibody staining of homozygous embryos with the neuron-specific antibody 22C10 (*15*) show no obvious morphological abnormalities of the nervous system (*10*), but they fail to hatch. Flies that survive to adulthood quickly become paralyzed when exposed to 29°C, and die rapidly; at 22°C, they die within 4 to 5 days (Fig. 3A). Death is preced-

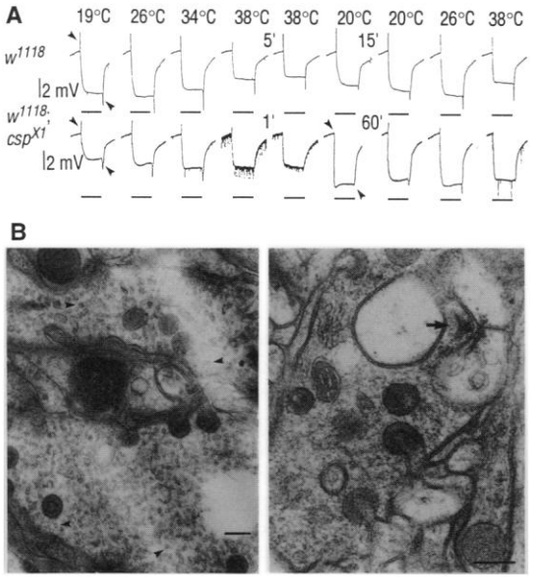


Fig. 4. Electroretinogram and EM of csp^{X1}. (**A**) ERGs of a w^{1118}; csp^+ (upper row) and a w^{1118}; csp^{X1} fly (lower row). The response contains a brief, positive "on" transient, a sustained gradually negative photoreceptor potential, and a brief "off" transient (arrowheads) (*16*). The flies were heated within 15 min from 19° to 38°C, then kept at 38°C (5 min for w^{1118}, 1 min for w^{1118}; csp^{X1}), cooled to 20°C within 2 min, and allowed to recover in the dark (15 min for w^{1118}, 60 min for w^{1118}; csp^{X1}). The cycle was then partially repeated. Note the disappearance of the transients after 1 min at 38°C in w^{1118}; csp^{X1} (arrowheads), but not in wild type, even after 5 min. Gradual recovery of the transients began after a few minutes of darkness at 20°C. The spikes superimposed on the 38°C traces of the mutant were from muscle tremors picked up by the indifferent electrode in the thorax. Scale: "Light on" intervals (0.6 s) are indicated by horizontal bars, amplitude by vertical bars (2 mV). Changing temperature is indicated on top. (**B**) Electron microscopy of synaptic terminals of photoreceptor axons in the lamina. Synaptic vesicles (arrowheads) were seen in wild type (left). In the csp^{X1} mutant (right), synaptic vesicles were difficult to discern, even at synaptic sites (arrow). The mutant synaptic terminals consistently showed electron-dense debris, which may represent degeneration products of vesicles. It remains unclear whether the number of vesicles is reduced or their mutant properties make them undetectable by the standard EM technique. At lower magnification, no morphological defect in the photoreceptor axons was evident. Note that both wild type and mutant were processed identically and simultaneously (*22*); this experiment was repeated several times with similar results. Bar, 0.2 μm.

ed by increasingly sluggish behavior, then intense, spasmic jumping, shaking, uncoordinated locomotion, and paralysis. The second mutant, csp^{X1}, with a smaller deletion, yielded more escapers (16%) at 25°C and was therefore more useful for further analysis. Immunoblots and immunohistochemistry of adults demonstrated some residual protein expression in csp^{X1} (Fig. 3, C and D), but not in csp^{R1}.

To test for physiological correlates of the ts paralytic behavior of csp^{X1}, we compared mutant and wild-type electroretinograms (ERGs) as the temperature was increased from 19° to 38°C. The mutant ERG showed a loss of "on" and "off" transients, which were restored by return to lower temperature (Fig. 4A). These transients derive from the response of first-order interneurons in the lamina and thus depend on synaptic transmission (*16*); their disappearance suggests blockage of synaptic transmission from photoreceptor terminals to lamina neurons.

To look for visible synaptic abnormalities in the photoreceptor terminals, we compared synaptic regions in the lamina of mutant csp^{X1} flies to those of wild type by electron microscopy (EM) (Fig. 4B). In contrast to wild type, synaptic vesicles in the mutant were hardly visible; the terminals were filled with unidentified fine, electron-dense "debris," possibly caused by neuronal degeneration. "Collard pit" structures at the terminal membranes, which would be suggestive of a block in endocytosis, as reported in the mutant *shibire* (*17*), were not observed.

Taken together, the synaptic vesicle localization of Tcsp in *Torpedo*, the ts behavioral and physiological phenotypes of *csp* mutants in *Drosophila*, and the effects of *Tcsp* mRNA on N-type Ca^{2+} channel activity indicate a role for these synaptic vesicle proteins in neurotransmitter release. Although the *csp* gene is not completely indispensable (some flies can survive its complete deletion), the block of synaptic transmission at higher temperatures indicates a possible requirement for the Csp proteins in stabilizing the components of the neurotransmitter release machinery. It is noteworthy that the entire deletion of *dnaJ*, a paralog of *csp* in *Escherichia coli*, exhibits a ts phenotype suggestive of chaperone activity (*18*). Mutants of the synaptic vesicle protein synaptotagmin (Syn) in *Drosophila* and *Caenorhabditis elegans* show phenotypic defects similar to those of *csp* mutants (*3*). They demonstrate an important requirement of the *syn* gene for proper synaptic function, but even null mutations do not completely eliminate neurotransmitter release (*3*). The Csp proteins could act as modulators or chaperones in synaptic vesicle formation, vesicle transport, or the assembly or regulation of multimeric protein complexes that interact with presynaptic membranes. Further studies with mutants affecting synaptic transmission may lead to a genetic dissection of the mechanisms.

REFERENCES AND NOTES

1. V. M. O'Connor, O. Shaotienko, E. Grishin, H. Betz, *FEBS Lett.* **326**, 255 (1993).
2. T. M. Jessell and E. R. Kandel, *Cell* **72**/*Neuron* **10** (suppl.), 1 (1993); R. B. Kelly, *ibid.*, p. 43; T. C. Südhof and R. Jahn, *Neuron* **6**, 665 (1991); P. Greengard, F. Valtorta, A. J. Czernik, F. Benfenati, *Science* **259**, 780 (1993); P. Thomas and W. Almers, *Curr. Opin. Neurobiol.* **2**, 308 (1992).
3. A. DiAntonio, K. D. Parfitt, T. L. Schwarz, *Cell* **73**, 1281 (1993); J. T. Littleton, M. Stern, K. Schulze, M. Perin, H. J. Bellen, *ibid.* **74**, 1125 (1993); M. L. Nonet, K. Grundahl, B. J. Meyer, J. B. Rand, *ibid.* **73**, 1291 (1993).
4. K. E. Zinsmaier *et al.*, *J. Neurogenet.* **7**, 15 (1990).
5. P. A. Silver and J. C. Way, *Cell* **16**, 5 (1993); A. J. Caplan, D. M. Cyr, M. G. Douglas, *Mol. Biol. Cell* **4**, 555 (1993); T. Kurihara and P. A. Silver, in *Membrane Biogenesis and Protein Targeting*, W. Neupert and R. Lill, Eds. (Elsevier, New York, 1992), pp. 309–327.
6. C. B. Gundersen and J. A. Umbach, *Neuron* **9**, 527 (1992).
7. A. Mastrogiacomo *et al.*, *Science*, **263**, 981 (1994).
8. A 0.73-kb fragment of type I cDNA was subcloned in frame with the glutathione-S-transferase of the expression vector pGEX-3 [D. B. Smith and K. S. Johnson, *Gene* **67**, 31 (1989)] and confirmed by sequencing. Large-scale expression of the fusion protein in *E. coli* and affinity purification of the fusion protein were as described [R. Reifegerste *et al.*, *J. Neurosci.* **13**, 2186 (1993)]. Monoclonal antibodies were generated (*15*) and screened by immunoblots.
9. J. T. Littleton *et al.*, *Development* **118**, 1077 (1993).
10. K. E. Zinsmaier, K. K. Eberle, E. Buchner, N. Walter, S. Benzer, data not shown.
11. A genomic *Drosophila* λ-"fix" library was screened [G. O. Pflugfelder *et al.*, *Genetics* **126**, 91 (1990)] with the cDNA lcz49-9 (*1*) by standard methods [J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989)]. Two phages (Lfix 9-8, Lfix 9-33) contained the entire transcribed region of the *csp* gene, shown in Fig. 2. The Sal I–Sal I double fragment from position −2.7 to +6.6 was sequenced. Comparison with the cDNA sequence allowed the mapping of the exon-intron structure and demonstrated the alternative splicing of the different transcripts.
12. P elements of the m-lac chromosome [E. Bier *et al.*, *Genes Dev.* **3**, 1273 (1989); stock provided by M. Brandt] were mobilized with transposase-providing ry^{506} *Sb* *P*[ry^{+} *Δ2-3*] element at 99B [H. M. Robertson *et al.*, *Genetics* **118**, 461 (1988)], which itself is unable to jump. Male progeny with successful insertions (pigmented eyes indicating a P element) were pooled and mated to w^{1118} females. After 3 days, the parents were transferred for overnight collection of eggs, from which genomic DNA (gDNA) was isolated [T. Laverty *et al*, *Rubin Lab Manual* (1986)]; the parents were saved. The gDNA was restricted with Eco RI and used for plasmid rescue as described [V. Pirrotta, in Drosophila, *a Practical Approach*, D. B. Roberts, Ed. (IRL, Oxford, Washington, DC, 1986), pp. 83–110], with the exception that electrotransformation was used (Bio-Rad *Gene Pulser*). The obtained colonies were pooled, and the DNA was isolated and dot-blotted for hybridization by standard techniques. The probe (*csp* genomic region; Hind III–Hind III double fragment, 12.3 kb) was labeled with digoxigenin (Dig) according to the manufacturer's instructions (Boehringer Mannheim). In case of a positive pool, the result was verified by Southern (DNA) blot and single fly lines established and retested.
13. Two strategies were used to induce deletions. In the first, the P element of csp^{P1} was remobilized by mating of homozygous females to ry^{506} *Sb* *P*[ry^{+} *Δ2-3*] [H. M. Robertson *et al.*, *Genetics* **118**, 461 (1988)]. In the second, csp^{P1} adult males were irradiated with gamma rays. In both cases, progeny were scored for loss of the P element by eye color (provided by the *white*$^{+}$ gene of the P[w^{+}, *lacZ*]-construct of csp^{P1}). Putative deletions were analyzed for gene rearrangements and modified protein expression.
14. The double Hind III fragment of 12.3 kb (Fig. 2A) was cloned into the pCaSpeR4 P-element vector to generate P[w^{+}, *csp*], and P-element transformation of w^{1118} flies was as described [A. C. Spradling, in Drosophila, *a Practical Approach*, D. B. Roberts, Ed. (IRL, Oxford, Washington, DC, 1986), pp. 175–198]. Transformed flies were crossed into the *csp* mutant background and made homozygous for the P insertion and the *csp* mutation.
15. S. L. Zipursky, T. R. Venkatesh, D. B. Teplow, S. Benzer, *Cell* **36**, 15 (1984).
16. L. W. Pak, in *Handbook of Genetics*, R. C. King, Ed. (Plenum, New York, 1975), pp. 703–733; P. E. Coombe and M. Heisenberg, *J. Neurogenet.* **3**, 135 (1986); P. E. Coombe, *J. Comp. Physiol.* **159**, 655 (1986).
17. J. H. Koenig and K. Ikeda, *J. Neurosci.* **9**, 3844 (1989).
18. R. Ohki, T. Kawamata, Y. Katoh, F. Hosoda, M. Ohki, *J. Biol. Chem.* **267**, 13180 (1992).
19. Frozen sections were fixed with PLP [I. W. McLean and P. K. Nakane, *J. Histochem: Cytochem.* **22**, 1077 (1974)] for 20 min, washed twice in 0.1 M phosphate-buffered saline (PBS), and incubated with hybridoma supernatant at a dilution of 1:10 in PBS/0.3% Triton X-100 for 1 hour at room temperature, then washed and incubated with horseradish peroxidase–conjugated goat antibody to mouse immunoglobulin (Bio-Rad) at 1:250 (fluorescein isothiocyanate–conjugated antibody 1:200) in PBS/0.3% Triton X-100. The sections were washed three times in tris-buffered saline [TBS; 130 mM NaCl, 5 mM KCl, 1 mM EGTA, 100 mM tris-HCl (pH 7.3)] and reaction products visualized with 0.05% diaminobenzidine and 0.002% hydrogen peroxide in TBS.
20. *Drosophila* polyadenylated [poly(A)$^{+}$] RNA was isolated as described [N. Bonini, W. M. Leiserson, S. Benzer, *Cell* **72**, 379 (1993)]. RNA gel electrophoresis was performed with 5 μg of wild-type head-, 5 μg of *eya* head-, and 10 μg of body-poly(A)$^{+}$ RNA by the high-resolution method [S. S. Tsang *et al.*, *BioTechniques* **14**, 380 (1993)]. Blotting and hybridization were done according to standard techniques.
21. Equal amounts of synaptosomal protein were assayed [M. M. Bradford, *Anal. Biochem.* **72**, 248 (1976)], separated by SDS–polyacrylamide gel electrophoresis, blotted, and immunostained as described (*1*), except that primary antibodies were diluted 1:5. Synaptosomal membranes were prepared from heads of flies frozen in liquid nitrogen and separated by sieving. Crude extracts were prepared by homogenization in 0.32 M sucrose, 5 mM Hepes-Na (pH 7.8), and conventional centrifugation as described [L. E. Kelly, *Comp. Biochem. Physiol.* **69B**, 61 (1981); B. Krueger, D. W. Ratzlaff, G. R. Strichartz, M. P. Blaustein, *J. Membr. Biol.* **50**, 287 (1979)].
22. I. A. Meinertzhagen and S. D. O'Neil, *J. Comp. Neurol.* **305**, 232 (1991).

23. We acknowledge the assistance of S. Ou and P. Patterson of the Caltech monoclonal antibody facility in providing the mAbs and E. Schwarz for the Northern blots. The "site-directed P-mutagenesis" was performed by two of us (K.K.E. and N.W.) in the laboratories of E. Hafen and M Noll at the University of Zurich. The technical assistance of M. Jenni, O. Zilian (Zurich), N. Krejci, and Y. Schleger of the Buchner laboratory and R. Young, L. Dowling, C. Pham, and E. Eichenberger of the Benzer laboratory is acknowledged. We thank the other members of the Benzer group for their critical comments on the manuscript. Supported by a fellowship to K.E.Z. and by grants to E.B. from the Deutsche Forschungsgemeinschaft and by grants to S.B. from the NSF, the National Eye Institute of the NIH, and the James G. Boswell Foundation.

16 September 1993; accepted 18 January 1994