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REGULAR ARTICLE

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Wide distribution of the cysteine string proteins in *Drosophila* tissues revealed by targeted mutagenesis

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Abstract The "cysteine string protein" (CSP) genes of higher eukaryotes code for a novel family of proteins characterized by a "J" domain and an unusual cysteine-rich region. Previous studies had localized the proteins in neuropil and synaptic terminals of larval and adult *Drosophila* and linked the temperature-sensitive paralysis of the mutants described here to conditional failure of synaptic transmission. We now use the null mutants as negative controls in order to reliably detect even low concentrations of CSPs by immunohistochemistry, employing three monoclonal antibodies. In wild-type flies high levels of cysteine string proteins are found not only in apparently all synaptic terminals of the embryonic, larval, and adult nervous systems, but also in the "tall cells" of the cardia, in the follicle cells of the ovary, in specific structures of the female spermatheca, and in the male testis and ejaculatory bulb. In addition, low levels of CSPs appear to be present in all tissues examined, including neuronal perikarya, axons, muscles, Malpighian tubules, and salivary glands. Western blots of isolated tissues demonstrate that of the four isoforms expressed in heads only the largest is found in non-neural organs. The wide expression of CSPs suggests that at least some of the various phenotypes of the null mutants observed at permissive temperatures, such as delayed development, short adult lifespan, modified electroretinogram, and optomotor behavior, may be caused by the lack of CSPs outside synaptic terminals.

Key words Exocytosis · "J" domain · Gene knock-out · Temperature-sensitive paralysis · Chaperone · Calcium channel · *Drosophila melanogaster* (Insecta)

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Introduction

Reverse genetics combined with gene targeting has become a major tool for the identification and functional characterization of novel proteins. In contrast to classical genetics this approach allows the isolation of mutants even if these display no detectable phenotype. It has been used successfully in bacteria, yeast, nematodes, and mice, and efficient variants have been developed for *Drosophila* (Ballinger and Benzer 1989; Kaiser and Goodwin 1990; Hamilton et al. 1991; Walter 1992; Guo et al. 1996). Here, we have employed gene targeting to study the function and distribution of a new family of synaptic vesicle-associated proteins, the cysteine string proteins (CSPs). cDNAs of the *Drosophila csp* gene were expression cloned using a monoclonal antibody (MAB ab49/DCSP1) (Zinsmaier et al. 1990). This MAB had been selected from a large hybridoma library raised against *Drosophila* heads due to its binding in immunohistochemical experiments to synaptic terminals in the *Drosophila* nervous system (Buchner et al. 1988; Hofbauer 1991). The gene has been mapped by in situ hybridization to 79E1–2 on chromosome 3 (Zinsmaier et al. 1990). The proteins inferred from three different cDNAs isolated so far (Zinsmaier et al. 1994) contain a "J" domain and an unusual sequence of 11 contiguous cysteine residues which were responsible for the naming of these proteins. The J-domain renders the CSPs members of the DnaJ protein family which has been conserved in evolution from *E. coli* to

Fig. 1 Primary structure of CSP isoforms derived from presently known genomic or cDNA sequences of various species. The "J" domain and the cysteine string are *single and double underlined*, respectively. Amino acids conserved in all species are marked by *asterisks*, those differing only in *C. elegans* by *pluses* (*CE Caenorhabditis elegans*, *D Drosophila melanogaster*, *T Torpedo californica*, *R* rat, *B* bovine, *H* human)

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CE-CSP    MNSDGLREAE EGRTSGGASP REESPAADHS HDPKKGLHLY NVL-IQKNAT
D-CSP1    M-SAPGMDKR KLSTSG---- ---------- ------DSLY EILGLPKTAT
D-CSP2    M-SAPGMDKR KLSTSG---- ---------- ------DSLY EILGLPKTAT
D-CSP3    M-SAPGMDKR KLSTSG---- ---------- ------DSLY EILGLPKTAT
T-CSP     M-GDQR--QR SLSTSG---- ---------- ------DSLY IVLGLDKNAS
R-CSP     M-ADQR--QR SLSTSG---- ---------- ------ESLY HVLGLDKNAT
B-CSP1    M-ADQR--QR SLSTSG---- ---------- ------ESLY HVLGLDKNAT
B-CSP2    M-ADQR--QR SLSTSG---- ---------- ------ESLY HVLGLDKNAT
H-CSP1    M-ADQR--QR-SLSTSG---- ---------- ------ESLY HVLGLDKNAT
H-CSP2    M-ADQR--QR-SLSTSG---- ---------- ------ESLY HVLGLDKNAT
          *        +  ++***                       +**   *++ * **

CE-CSP    DDEIKKAYRK LALRYHPDKN LDGDPEKTEM FKEINYANAV LSNPNKRRVY
D-CSP1    GDDIKKTYRK LALKYHPDKN PD-NVDAADK FKEVNRAHSI LSDQTKRNIY
D-CSP2    GDDIKKTYRK LALKYHPDKN PD-NVDAADK FKEVNRAHSI LSDQTKRNIY
D-CSP3    GDDIKKTYRK LALKYHPDKN PD-NVDAADK FKEVNRAHSI LSDQTKRNIY
T-CSP     PEDIKKSYRK LALKYHPDKN PD-NPEASEK FKEINNAHAI LTDATKRNIY
R-CSP     SDDIKKSYRK LALKYHPDKN PD-NPEAADK FKEINNAHAI LTDATKRNIY
B-CSP1    SDDIKKSYRK LALKYHPDKN PD-NPEAADK FKEINNAHAI LTDATKRNIY
B-CSP2    SDDIKKSYRK LALKYHPDKN PD-NPEAADK FKEINNAHAI LTDATKRNIY
H-CSP1    SDDIKKSYRK LALKYHPDKN PD-NPEAADK FKEINNAHAI LTDATKRNIY
H-CSP2    SDDIKKSYRK LALKYHPDKN PD-NPEAADK FKEINNAHAI LTDATKRNIY
            +*** *** ***+****** +* + ++  + *** * *+ + * + +**++*

CE-CSP    D-MGETGLKL MEQFGEDEKI LQWMLKPWFK WTFFAF-GLL TGGFFCCCCG
D-CSP1    DNYGSLGLYI AEQFGEENVN AYFVVTSPAV KAVVICCAVI T---GCCCCC
D-CSP2    DNYGSLGLYI AEQFGEENVN AYFVVTSPAV KAVVICCAVI T---GCCCCC
D-CSP3    DNYGSLGLYI AEQFGEENVN AYFVVTSPAV KAVVICCAVI T---GCCCCC
T-CSP     DKYGSLGLYV AEQFGEENVN TYFVLSSWWA KALFVFCGVI T---GCYFCC
R-CSP     DKYGSLGLYV AEQFGEENVN TYFVLSSWWA KALFVVCGLL T---CCYCCC
B-CSP1    DKYGSLGLYV AEQFGEENVN TYFVLSSWWA KALFIFCGLL T---CCYCCC
B-CSP2    DKYGSLGLYV AEQFGEENVN TYFVLSSWWA KALFIFCGLL T---CCYCCC
H-CSP1    DKYGSLGLYV AEQFGEENVN TYFVLSSWWA KALFVFCGLL T---CCYCCC
H-CSP2    DKYGSLGLYV AEQFGEENVN TYFVLSSWWA KALFVFCGLL T---CCYCCC
          * +*++**+  +*****++++  +++  +    ++    +    *    *  *+

CE-CSP    CMCCCQCCCN CCGKYKPKHD DEFADETSDG DVIVDQPTAS EPMPDTNNRQ
D-CSP1    CCCCCCNF-- CCGKFKPPVN -ESHDQYSHL NRPDGNREGN DMPTHLGQPP
D-CSP2    CCCCCCNF-- CCGKFKPPVN -ESHDQYSHL N--------- ----------
D-CSP3    CCCCCCNF-- CCGKFKPPVN -ESHDQYSHL N--------- ----------
T-CSP     CLCCCCNC-- CCGKCKPKPP -EGEEQEYYV SPEDLEAQLQ SDMEKEGDGA
R-CSP     CLCCCFNC-- CCGKCKPKAP -EGEETEFYV SPEDLEAQLQ SDEREATDTP
B-CSP1    CLCCCFNC-- CCGKCKPKAP -EGEETEFYV SPEDLEAQLQ SDEREAADTP
B-CSP2    CLCCCFNC-- CCGKCKPKAP -EGEETEFYV SPEDLEAQLQ SDERGGH
H-CSP1    CLCCCFNC-- CCGKCKPKAP -EGEETEFYV SPEDLEAQLQ SDEREATDTP
H-CSP2    CLCCCFNC-- CCGKCKPKAP -EGEETEFYV SPEDLEAQLQ SDERGGH
          * *** +    **** **     *

CE-CSP    VPI------- ---------- ---------- -VIAMPPPPS QKD
D-CSP1    RLEDVDLDDV NLGAGGAPVT SQPREQAGGQ PVFAMPPPSG AVGVNPFTGA
D-CSP2    --EDVDLDDV NLGAGGAPVT SQPREQAGGQ PVFAMPPPSG AVGVNPFTGA
D-CSP3    --EDVDLDDV NLGAGGAPVT SQPREQAGGQ PVFAMPPPSG AVGVNPFTGA
T-CSP     ---------- ---------- ---------- ---------- ------IVVQ
R-CSP     ---------- ---------- ---------- ---------- ------IVIQ
B-CSP1    ---------- ---------- ---------- ---------- ------IVIQ
H-CSP1    ---------- ---------- ---------- ---------- ------IVIQ

D-CSP1    PVAANENTSL NTTEQTTYTP DMVNQKY
D-CSP2    PVAANENTSL NTTEQTTYTP DMVNQKY
D-CSP3    PVAANENTSL NTTEQTTYTP GI
T-CSP     PTSATETTQL TSDSHPSYHT E
R-CSP     PASATETTQL TADSHPSYHT DGFN
B-CSP1    PASATETTQL TADSHPSYHT DGFN
H-CSP1    PASATETTQL TADSHPSYHT DGFN
          +  + + + +        +
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man (Silver and Way 1993). While the exact functions of most members of the DnaJ family are unclear, some are known to activate heat shock proteins implicated in molecular chaperone functions such as aiding or inhibiting protein folding or oligomerization. Interest in brain chaperones has recently grown due to a possible link between errors in protein folding and neurodegenerative brain disease (Welch and Gambetti 1998).

Independently, a cDNA for a vertebrate homologue of the *Drosophila* CSPs has been cloned from *Torpedo* as part of an effort to identify functional components of presynaptic calcium channels (Gundersen and Umbach 1992). By homology screening, cDNAs for rat, bovine, and human CSPs (Fig. 1) were identified (reviewed by Buchner and Gundersen 1997). It was also established that *Torpedo*, rat, and *Drosophila* CSPs from neural tissues cofractionate with synaptic vesicles (Mastrogiacomo et al. 1994; Zinsmaier et al. 1994; van de Goor et al. 1995; Mastrogiacomo and Gundersen 1995; van de Goor and Kelly 1996), and it was shown that vertebrate CSPs are widely distributed in both neural and non-neural tissues (Braun and Scheller 1995; Kohan et al. 1995; Chamberlain and Burgoyne 1996; Coppola and Gundersen 1996: Pupier et al. 1997). Moreover, at least 11 of the 13 cysteines of *Torpedo* CSPs are modified posttranslationally by palmitoylation, rendering the CSPs highly amphipathic (Gundersen et al. 1994).

In order to obtain information on the function of the *Drosophila* CSPs in vivo we have generated several types of mutants in the *csp* gene. Analysis of electroretinograms (Zinsmaier et al. 1994) and recordings from larval nerve-muscle preparations (Umbach et al. 1994) have demonstrated that paralysis of *csp* mutants at high temperatures is caused by a block of synaptic transmission due to disrupted presynaptic depolarization-secretion coupling. At permissive temperatures the time course of quantal release measured at single synaptic boutons was found to be disturbed in the mutants (Heckmann et al. 1997). Here we report the molecular defects of the mutants, describe several phenotypic traits that are observed at "permissive" temperatures, and study the distribution of CSPs in wild-type embryos, larvae, and adults by immunohistochemistry using the null mutants as negative controls in order to discriminate between specific and unspecific staining.

Materials and methods

Transposon mutagenesis and generation of deficiencies by γ-ray irradiation

The mutagenesis procedure employing the P element transposon P(w^+) (Bier et al. 1989) has been described in detail elsewhere (Walter 1992). By this procedure two P-insertion strains, csp^{P1} and csp^{P2}, were isolated from 24 000 transposition events as outlined previously (Zinsmaier et al. 1994). The w^+ gene of these P insertions allows the detection of chromosomal deficiencies affecting the region of the *csp* gene by eye color inspection. Using a γ-radiation dose of 60 Gy that in a biological calibration experiment had induced in about 50% of the X-chromosomes a hemizygous lethal mutation, 150 homozygous csp^{P1} males (age 3 days) were irradiated and 24 h later crossed to 450 homozygous csp^{P1} virgin females (age 3–4 days). The selection criterion in the F_1 generation was an eye color different from homozygous csp^{P1}. Each F_1 fly that deviated in eye color from csp^{P1}/csp^{P1} was crossed individually with three to four w^{1118};*TM1/TM3* flies of the opposite sex to establish balanced or, if viable, homozygous stocks. From 6300 F_1 offspring of γ-irradiated csp^{P1} flies, 28 balanced lines with reduced or missing w^+ expression were established, 14 of which were homozygous lethals at 18°C. The 14 homozygous lines viable at 18°C were examined for CSP expression in Western blots and immunohistochemistry. Two lines, csp^{X1} and csp^{X2}, showed no recognizable trace of specific labeling in Western blots with antibodies against *Drosophila* CSP. Both strains proved lethal at elevated temperatures (25°C). In addition, they displayed a temperature-sensitive paralytic phenotype and were therefore used for screening various mutagenized chromosomes for non-complementation of CSP deficiency. This identified five additional *csp* alleles among the lines that were lethal even at 18°C (csp^{X3}, through csp^{X7}). However, since lethality at 18°C, but not lethality at 25°C and temperature-sensitive paralysis, complemented in most mutual crosses between these lines, the possibility that unconditional lethality might be due to different second site mutations, independent of the *csp* gene, had to be considered. In addition, analysis of the csp^{X1} and csp^{X2} mutants indicated that they were not molecular null alleles (see below). Thus, it was not clear whether residual CSP fragments were responsible for viability of csp^{X1} and csp^{X2} at 18°C. We therefore devised a mutagenesis scheme that could generate null alleles by the precise deletion of the *csp* gene without likely induction of second site mutations.

Precise deletion of the *csp* gene by heat shock-enhanced recombination

In order to obtain null alleles without likely second site mutations, we made use of the (syndirectionally oriented) transposon insertions immediately upstream and downstream of the *csp* gene in the lines csp^{P1} and csp^{P2}, respectively. In csp^{P1}/csp^{P2} trans-heterozygotes, meiotic pairing and crossover within the paired two P elements can result in a precise deletion of the region between the two P elements which contains the *csp* gene, with only one hybrid P element left behind. Female P_1/P_2 transheterozygotes were crossed to homozygous csp^{X1} males. Tests were made on 150 000 offspring raised at 18°C for non-complementation of the csp^{X1} phenotype (temperature-sensitive paralysis). Since the *csp* gene lies close to the centromere (79E1–2), where crossover probability is very low (Ashburner 1989a), heat treatment (34°–36°C for 17–23 h during stages P10–P15 of pupal development) was applied in order to increase the number of recombination events (Grell 1978). Flies that were paralyzed within 10 min at 37°C were returned to 18°C immediately, and individually crossed to w^{1118};*TM* partners. Balanced and, if viable, homozygous lines were established by standard procedures. Of 15 non-sibling flies isolated in this manner, 11 balanced lines were established (4 flies produced no offspring). Two lines spontaneously reverted to wild type; 5 were homozygous lethal at 18°C and did not complement the lethality of the csp^{X3} allele. The remaining four homozygous viable lines were termed csp^{U1} through csp^{U4}.

Jump-out mutagenesis

Additional deletions in the *csp* gene were generated by mobilization of the P element in the csp^{P2} strain as outlined previously (Zinsmaier et al. 1994). Individual crosses were made of 465 male $csp^{P2}/\Delta 2$–3Sb flies to w^{1118}/w^{1118}; *TM3 Ser/TM6 D* females. From the offspring of each line a single male with w^- eyes (w^{1118}; *P2 jumpout/TM* or w^{1118}; *P2 del/TM*) was used to establish a genetically balanced stock. This ensured that all studied excisions were independent. Of the 345 excision strains generated, 322 were homozygous viable at 25°C and 23 were homozygous lethal at this temperature. By Southern blotting, we found that 257 (75%) had suffered a precise excision of *P2*, 49 (14%) apparently had only a small defect in the *mini-* w^+ gene not visible in the blots, and 39 (11%) contained an

imprecise excision of the P element. Eight of the 39 imprecise excision strains belonged to the homozygous lethal group. (The remaining 15 lethal strains were precise excisions indicating that they contained lethal mutations elsewhere on the chromosome.) From the eight lethal imprecise excisions, seven showed deletions reaching into the 3' end of the *csp* gene, but only four of these failed to genetically complement the csp^{X1} allele. Unfortunately, two of these were lost. The alleles csp^{R1} and csp^{R2} were kept for further analysis.

Other experimental techniques

Genetic analysis, immunohistochemistry, Western blotting, molecular characterization of deficiency chromosomes, electroretinogram recordings, and behavioral analysis were used as described previously or standard procedures were used (Pak 1975; Buchner et al. 1986; Harlow and Lane 1988; Ashburner 1989a,b; Sambrook et al. 1989; Lindsley and Zimm 1992; Melzig et al. 1996). For ultrastructural localization of CSPs third instar larvae were dissected in *Drosophila* Ringer's solution, fixed for 12 h in 4% paraformaldehyde, dehydrated and embedded in LRwhite. Thin sections were immunostained using MAB ab49/DCSP1 and gold-labeled anti-mouse antiserum (Dianova, West Grove, Pa., USA).

Results

Molecular analysis of the *Drosophila csp* mutants

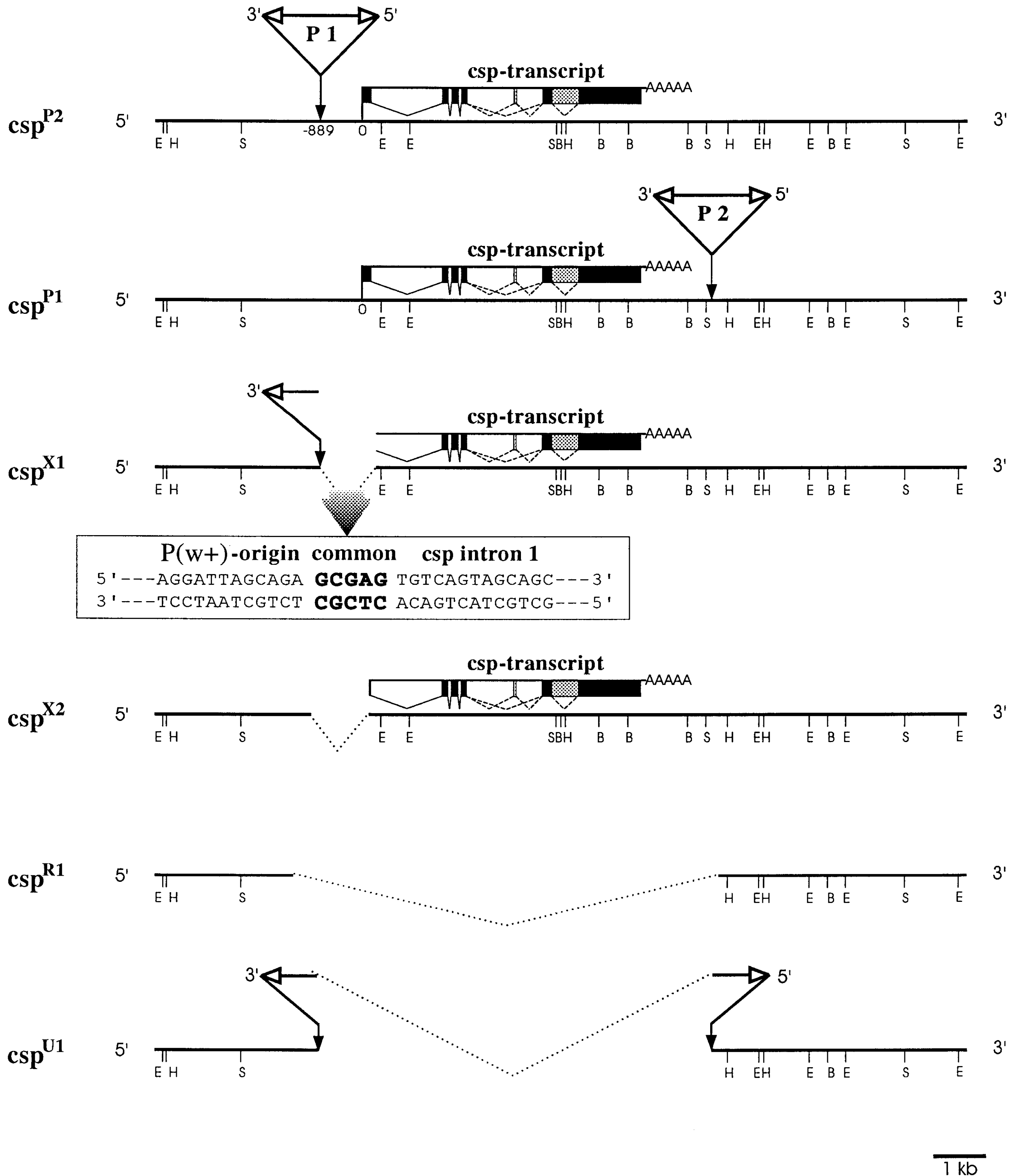
We sequenced 10.5 kb of the wild-type genomic region containing the *csp* gene (GenBank accession #AF057167) in order to verify the cDNA sequences, to determine the exact exon-intron organization of the gene, and to allow the identification of mutant chromosomal breakpoints (Fig. 2). Comparison with the genetic database disclosed the identity of the gene 5' adjacent to *csp* that was detected earlier by Northern blots and mutant complementation analysis (Zinsmaier et al. 1994). The first 1084 bp of the above sequence contain exonic sequences and a small intron of the *Ddx1* gene that codes for a homologue of the human DEAD-box gene *DDX1* (Rafti et al. 1996).

Three protein isoforms can be derived from the known cDNAs (Zinsmaier et al. 1994). The CSP1 isoform (D-CSP1 in Fig. 1) contains a domain of 21 amino acids encoded by exon 5 that is deleted in CSP2 and CSP3 by differential RNA splicing. CSP2 and CSP3 differ only in seven amino acids at the C terminus due to the retention of intron 6 in the cDNA encoding CSP3. Comparison of the amino acid sequence of the *Drosophila* CSP isoforms with homologous proteins of other species reveals the high conservation of the "J" domain (underlined in Fig. 1) and the cysteine string (double underlined) from *Caenorhabditis elegans* to man. Significant homology is also found in the domain separating "J" and the cysteine string.

Targeted mutagenesis in *Drosophila* requires as a first step the isolation of flies carrying a P transposon insertion within or in close proximity to the gene under study. In an extensive P mutagenesis screen two independent $P(w^+)$ transposon insertion strains were isolated as described previously (Walter 1992; Zinsmaier et al. 1994). Figure 2 illustrates the positions of the two insertions upstream and downstream of the *csp* transcription unit in the strains csp^{P1} and csp^{P2}, respectively. Homozygous csp^{P1} or csp^{P2} flies show normal *csp* expression and exhibit no recognizable phenotype. Interestingly, they also fail to produce the enhancer trap lacZ expression expected for $P(w^+)$ insertions near a gene that is strongly expressed in various tissues (see below), suggesting that *csp* enhancers, if present, do not interact with the heat shock promoter of the $P(w^+)$ construct. In order to obtain defects in the *csp* gene, we generated deficiencies by γ-irradiation (X-alleles), $P(w^+)$ remobilization (R-alleles), or by a scheme of heat-enhanced unequal crossing-over (U-alleles), as described in "Materials and methods."

Southern blots probed with fragments from the *csp* genomic walk and sequencing of plasmid rescue fragments or polymerase chain reaction (PCR)-amplified segments identified the size and location of the chromosomal deficiencies. The maps of four deletion chromosomes are shown in Fig. 2. In two strains, the entire region of the *csp* gene between the two P-insertions has been eliminated, in csp^{R1} by a fortuitous event of imprecise excision of the remobilized transposon of csp^{P2}, in csp^{U1} by unequal crossing-over between the two P-insertions in the germline of a csp^{P1}/csp^{P2} trans-heterozygous female. This latter process leaves a hybrid $P2/1(w^+)$ behind (cf. Fig. 2). In the γ-irradiated strains csp^{X1} and csp^{X2}, the deficiencies eliminate upstream sequences of the *csp* gene and a small part of the 5' end of the transcription unit. Sequencing showed that in csp^{X1} the first exon, which codes for the 12 N-terminal amino acids, has been deleted. The identity of 5 bp in $P(w^+)ori$ and the first *csp* intron (cf. box in Fig. 2) may have been instrumental in generating the deletion at this position. Southern blots of csp^{X2} and the unconditionally lethal lines csp^{X3} through csp^{X7} revealed that in csp^{X3} the entire locus has been deleted, while the remaining five lines displayed the same modified Southern

Fig. 2 Exon-intron structure of the *csp* gene and genomic maps of the *csp* mutants. Comparison of 10 519 bp of genomic sequence (GenBank accession #AF057167) containing the *csp* gene with known cDNA sequences revealed that nucleotides (nt) 1–397 and nt 470–1084 belong to the last two exons of the *Ddx1* gene (accession #U34773). The *csp* transcript contains seven exons (nt 2654–2881; 4453–4592; 4674–4826; 4889–5010; 6041–6103; 6687–6891; 7498–8874). *Solid bars* of the *transcripts* represent exons found in all cDNAs; differentially spliced exons are *stippled*. All exon-intron boundaries conform to the GT-AG rule. The two P-element insertions csp^{P1} and csp^{P2} 3' to nt 1805 and 10519, respectively, were generated by site-selected P-mutagenesis. csp^{X} alleles were obtained by X- or γ-ray irradiation of csp^{P1} flies. In the mutant csp^{X1} the sequence from nt 1806 through nt 2966 has been replaced by a short 3' fragment of the $P(w^+)$ element. The stretch of 5 nt (nt 2967–2971) common to both the first intron and the bacterial *ori* of the residual P fragment (*inset*) may have played a role in creating the breakpoints of the csp^{X1} deletion. The exchange of a C for a G at nt 2975 in csp^{X1} may represent a point mutation, a polymorphism, or a cloning/sequencing artifact. csp^{R} alleles were produced by remobilization of the P-transposon of csp^{P2} flies. csp^{U} alleles were obtained after heat-enhanced unequal recombination between the csp^{P1} and csp^{P2} chromosomes in trans-heterozygotes. In the mutant csp^{U1} the sequence from nt 1806 through nt 10519 has been replaced by the (presumably intact) $P(w^+)$ element



pattern as csp^{X1}. Since the deficiency in csp^{X3} extends 5' and 3' beyond the cloned *csp* genomic region, lethality at 18°C of this strain must be expected due to the loss of the neighboring vital *Ddx1* gene (and possibly additional deleted genes) and does not result from the loss of *csp*. The lines csp^{X4}, csp^{X5}, and csp^{X7} mutually complement lethality but not the csp^{X1} phenotype; they are therefore assumed to have suffered a similar small deletion as csp^{X1}, but in addition received different lethal second site mutations. In csp^{X6}, apparently the same second site as in csp^{X3} has been hit. The csp^{U1} strain represents a true molecular *csp*-null allele. In order to be able to quantitatively compare visual function in (white-eyed) hypomorphs csp^{X1}, the null mutation csp^{U1}, and the (white-eyed)

Table 1 Summary of *csp* mutants, their defects and phenotypes (*del.* deletion, *ssm* second site mutation, *ts* temperature-sensitive, *wild type* no difference to wild type detected, *nd* not determined)

Mutant	Defect	Phenotype	Mutant	Defect	Phenotype
csp^{P1}	P-insertion 5'	wild type	csp^{U1}	Gene replaced by P element	ts-paralysis
csp^{P2}	P-insertion 3'	wild type	csp^{U1w}	Precise excision	ts-paralysis
csp^{X1}	Small deletion	ts-paralysis	csp^{U2}	nd	ts-paralysis
csp^{X2}	Small deletion	ts-paralysis	csp^{U3}	nd	ts-paralysis
csp^{X3}	Large deletion	lethal	csp^{U4}	nd	ts-paralysis
csp^{X4}	Small del.+ssm	lethal	csp^{R1}	Precise excision	ts-paralysis
csp^{X5}	Small del.+ssm	lethal	csp^{R2}	Inversion	lethal
csp^{X6}	Small del.+ssm	lethal			
csp^{X7}	Small del.+ssm	lethal			

w^{1118}; csp^{+} used to generate the mutants, we eliminated the hybrid P2/1(w^{+}) transposon from csp^{U1} by induction of transposition as described in "Materials and methods" and screening for white-eyed offspring. In this way, the white-eyed variant csp^{U1w} was generated. The alleles csp^{U2} through csp^{U4}, not yet analyzed in detail, will be described elsewhere. csp^{R2} has suffered an inversion with one breakpoint within the *csp* gene; the other beakpoint has not been characterized. Since this strain is lethal also at 18°C, it is assumed that the second breakpoint destroys a vital function independent of the *csp* gene. Table 1 summarizes molecular defects and phenotypes of the *csp* mutants isolated so far.

Mutant phenotypes

The temperature-sensitive paralysis and short adult life of csp^{X1} and csp^{R1} mutants at elevated temperatures has been described and analyzed previously (Umbach et al. 1994; Zinsmaier et al. 1994). Here we report defects of the mutants that are apparent already at the "permissive" temperature of 18°C.

Delayed development

The molecular null mutations csp^{U1} and csp^{R1} are homozygous viable at 18°C, demonstrating that, at this temperature, CSPs are not required for survival. Quantitative evaluation of csp^{U1} cultures shows that the mutants develop more slowly than wild type (Fig. 3A) but that viability of embryos, larvae or pupae is not significantly reduced. Adult flies at 18°C, however, have a severely reduced life span (Fig. 3B), during which they lay fewer eggs (20–70% of wild type, depending on experimental conditions). Towards the end of their life, they display a wide variety of neurological symptoms, such as uncoordinated movement, staggering, shaking, and spasmic extension of hind legs, before they become paralyzed. At 23°C or higher, viability is dramatically reduced at all stages beyond late embryo. At this temperature, only a few escapers survive to adulthood and stocks cannot be maintained. Temperature shift to the restrictive range after adult eclosion causes the described neurological symptoms and death over a period of a few minutes to days (Fig. 3B), depending on the temperature. If heat exposure is sufficiently short, these symptoms are reversible upon return to 18°C. The strains csp^{X1} or csp^{X2} show similarly reduced viability and the same temperature-dependent paralytic phenotype as the molecular null mutants. Apart from some residual immunohistochemical staining described below for csp^{X1} and csp^{X2}, no differences in phenotype between these two mutants and the null mutant csp^{U1} have so far been observed such that, for all practical purposes, csp^{X1} and csp^{X2} can be considered to be functional null alleles.

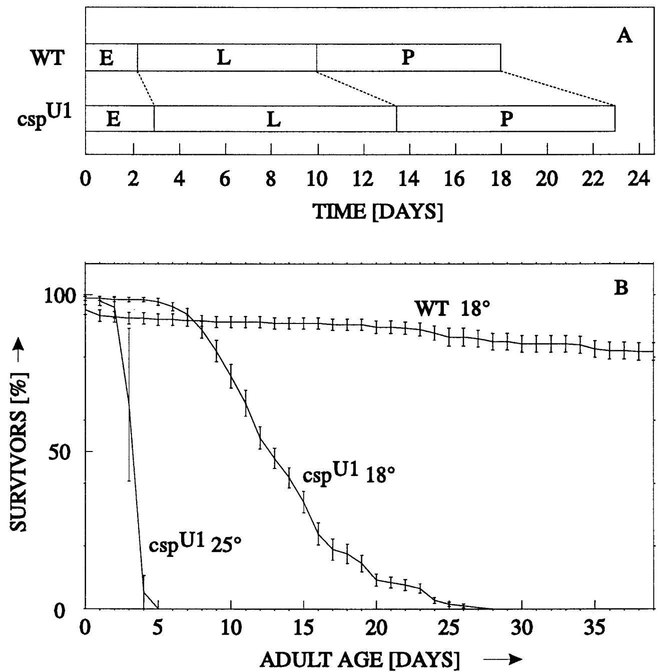


Fig. 3 **A** Developmental times of wild type (w^{1118}; csp^{+}) and mutant (w^{1118}; csp^{U1}) embryos (*E*), larvae (*L*), and pupae (*P*) at 18°C. Egg deposition defines time zero. The mutant develops more slowly in all three stages. **B** Survival vs age of wild type and csp^{U1} mutants at 18°C and 25°C. Each curve was obtained with at least 160 flies in three to five vials. Survival of wild type at 25°C (not shown) was 91% at 25 days

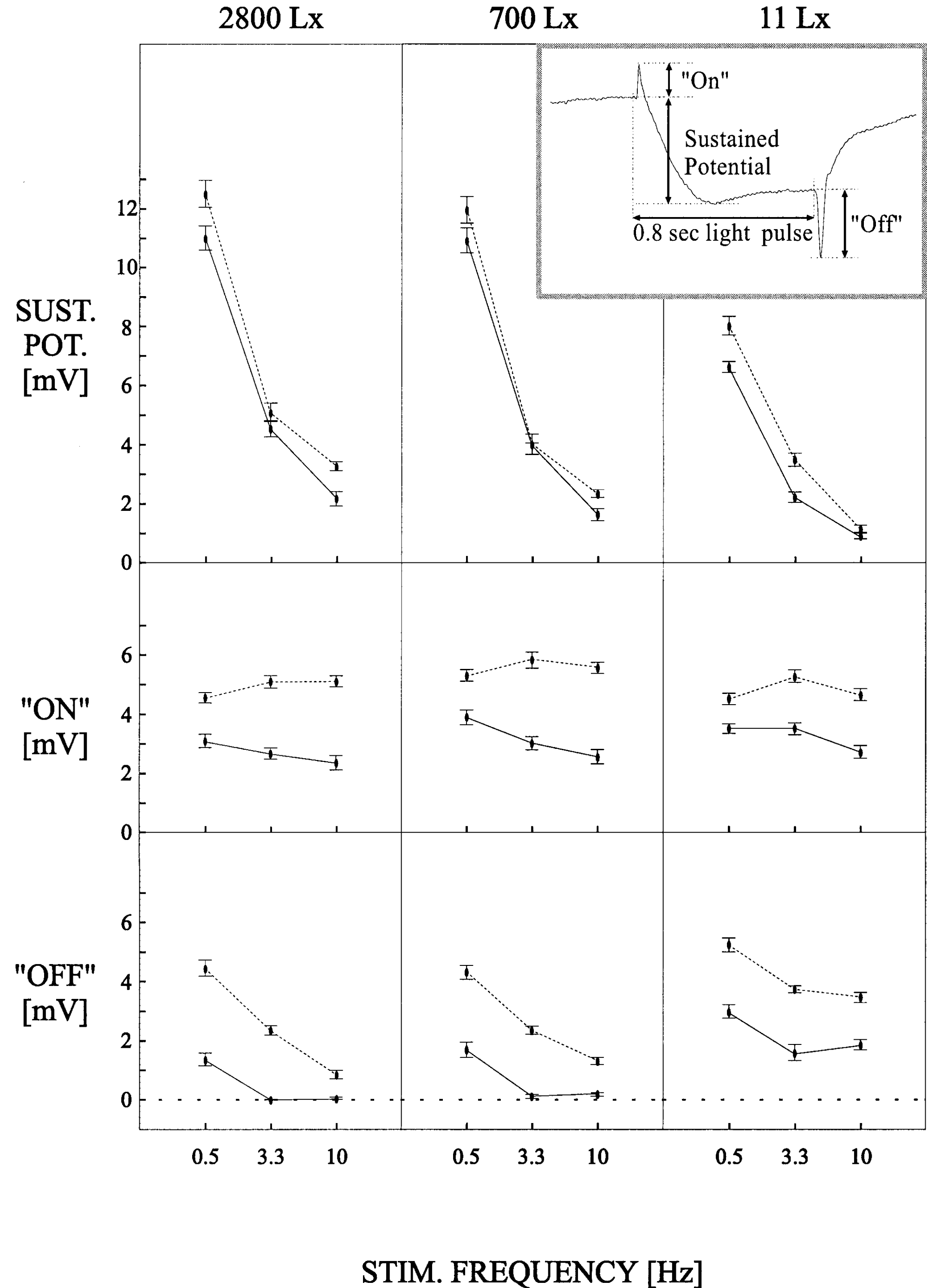


Fig. 4 Dynamic properties of electroretinogram (ERG) defects in *csp*^{X1} mutants. ERG responses to periodic light on/off signals can be broken down into three components as indicated in the *inset* (*horizontal bar* indicates "light on"). The "sustained potential" (*SUST. POT.*) represents an extracellular manifestation of the photoreceptor depolarization. "ON" transients and "OFF" transients arise from first-order interneurons of the lamina, reflecting synaptic transmission between photoreceptors and these interneurons. *w*^{1118}; *csp*^{X1} (*solid line*) and *w*^{1118}; *csp*$^{+}$ (*dashed line*) responses were recorded at 18.5°C and are given as functions of stimulus repetition frequency at three different light intensities. The mutant shows only minor impairment of the "sustained potential" (*upper panels*). "ON" responses are significantly reduced in the mutant (*middle panels*). Mutant "OFF" responses become vanishingly small at 3.3 and 10 Hz (*lower panels*) but appear to recover at the lowest light level. Light on:off periods were 1:1 except at 0.5 Hz (0.8:1.2). Each data point represents the average of at least 80 evaluated ERGs recorded from 6 flies. *Error bars* SEM

Electrophysiology

Since immunohistochemistry had localized the CSPs to synaptic terminals of presumably all neurons, a first qualitative test of synaptic function in the mutant *csp*^{X1} was carried out by ERG recordings (Zinsmaier et al. 1994). Here we extend these experiments to quantitatively compare luminance and frequency dependence of "on" and "off" transients of this summed potential for (white-eyed) *csp*$^{+}$ and the (white-eyed) mutant *csp*^{X1}, in order to obtain information on dynamic aspects of the role of CSP in synaptic function at permissive temperatures (18.5°C). The ERG transients are known to derive from electrical signals in first order visual interneurons (LMCs) (Heisenberg 1971) and thus reflect the properties of synaptic transmission from the photoreceptors to LMCs. The sustained component of the ERG essentially represents the summed photoreceptor response and thus should be largely independent of synaptic transmission. Figure 4 summarizes the evaluation of ERG recordings at the three different luminance levels indicated, from white-eyed *w*^{1118}; *csp*^{X1} and, as a control, *w*^{1118}; *csp*$^{+}$ flies, the strain from which

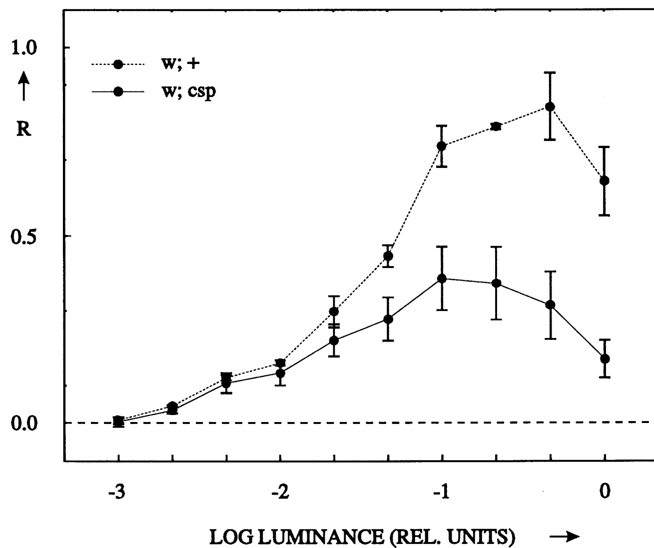


Fig. 5 Turning responses of 2- to 8-day-old walking flies at 18°C elicited by moving gratings as a function of pattern luminance. No difference between the *csp* null mutant w^{1118}; csp^{U1w} (*solid line*, 242 measurements from 9 flies) and the wild-type control w^{1118}; csp^{+} (*dashed line*, 80 measurements from 2 flies) is observed at low luminance but relative impairment of the mutants increases with increasing light levels. *Error bars* SEM

the *csp* mutants were derived. As expected, no dramatic difference was observed for the sustained ERG component. The "on" transient, however, is generally smaller in the mutant, and impairment increases at high stimulus frequencies. The "off" transient, on the other hand, disappears entirely at high and intermediate luminance levels when the light pulse frequency is increased, but is less severely affected at low light intensities.

Behavioral analysis of csp^{U1w} *mutants*

Measurements of luminance threshold for optomotor responses represent a sensitive, quantitative assay for overall performance of the adult *Drosophila* nervous system (Heisenberg and Götz 1975). At permissive temperatures (18°C), we compared the turning responses to a rotating striped drum of white-eyed flies lacking all CSP isoforms (w^{1118}; csp^{U1w}) with normal white-eyed animals (w^{1118}; csp^{+}) (Fig. 5). Surprisingly, at low luminance levels, responses of mutant and wild type were not significantly different, indicating that at 18°C CSPs are dispensable for the entire stimulus response chain, including light reception, direction-sensitive visual movement detection, spatial integration, transmission of the information to the motor system, and motor performance. At higher light levels, however, turning responses of the mutants are reduced by more than 50% and appear to be more variable.

Expression of CSPs in *Drosophila* tissues

Earlier in situ hybridization experiments and immunohistochemical stainings had demonstrated that *csp* transcripts are predominantly found in retina and cellular cortex of the adult brain, and that CSP-like immunoreactivity is concentrated in brain neuropil and synaptic boutons on larval and adult muscles (Zinsmaier et al. 1990, 1994). Here, we compare immunohistochemical preparations from wild type and the null mutant csp^{U1}, in order to reliably discriminate CSP immuno-labeling from unspecific cross-reactivity of the antibodies. It thus becomes possible to identify even weak CSP-specific staining. Since in preliminary experiments MABs ab49/DCSP1 and DCSP2 had always produced qualitatively identical staining patterns in wild-type preparations, only MABs ab49/DCSP1 and DCSP3 were used here for the detailed analysis. The salient features of CSP distribution are shown in Figs. 6–8.

Adult nervous system

Head sections from wild type (Fig. 6, left column) and mutant (middle column) were stained with MAB ab49/DCSP1 on the same microscope slide to warrant identical histochemical treatment, and were subjected to identical photography. Thus, even low concentrations of diaminobenzidine (DAB) reaction product found in wild type but not in the equivalent mutant structures, demonstrate the presence of CSPs. As noted earlier, high concentrations of CSPs are seen in synaptic boutons of motor neurons (Fig. 6K, arrows) and in all neuropil regions, illustrated here for lamina, medulla, and lobula complex of the visual system (D), and for mushroom bodies, ellipsoid body, fan-shaped body, and lateral protocerebrum of the central brain (G). In addition, however, low but significant levels of CSPs are present in the retina and in the cellular rind of the brain (compare Fig. 6A,D,G with B,E,H, respectively). A similar concentration difference of CSPs in neuropil and cellular rind is observed in the thoracic and abdominal ganglia (not shown).

Immunohistochemical staining with MAB DCSP3, which recognizes only two of the four known CSP isoforms (Fig. 9 and Zinsmaier et al. 1994), results in a generally reduced intensity of labeling, but all structures that show strong, specific staining with MAB ab49/DCSP1 also specifically bind MAB DCSP3 (right column of Fig. 6). This was again verified by staining sections of wild type and the null mutant csp^{U1} on the same microscope slide. However, comparison of Fig. 6A,D,G with C,F,I, respectively, illustrates a distinct difference in the neuropil staining patterns of the two antibodies: while MAB ab49/DCSP1 apparently equally binds to all synapses, resulting in relatively homogeneous neuropil staining, MAB DCSP3 preferentially labels synapses of photoreceptors (R1–8) and of selected sets of neurons, producing prominent staining of the lamina (terminals of R1–6), several layers in the medulla (including R7/8 terminals) and

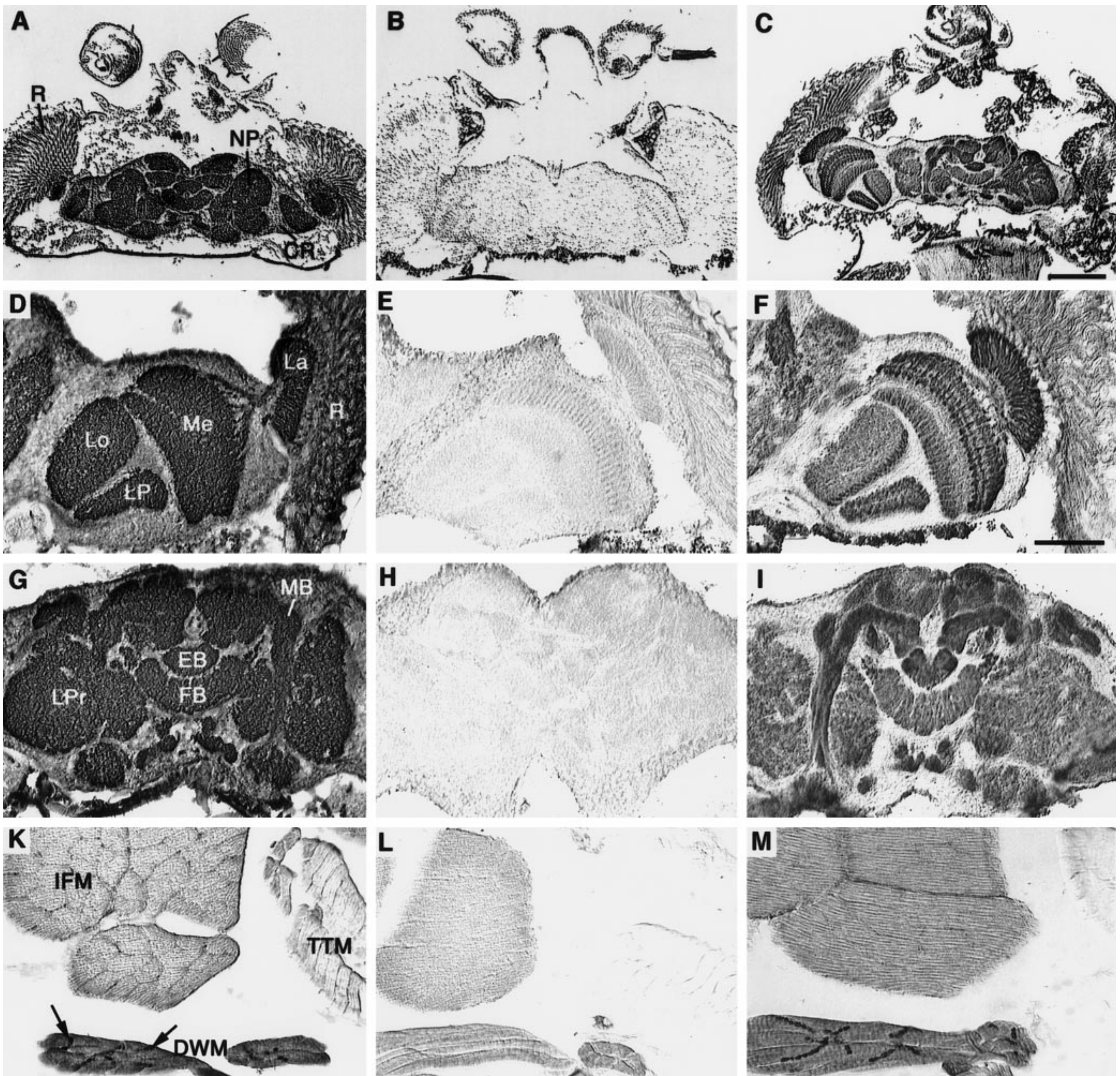


Fig. 6 Distribution of CSPs in the adult nervous system of *Drosophila*. Wild-type frozen sections were immunohistochemically labeled using MAB ab49/DCSP1 (*left column*) and MAB DCSP3 (*right column*). MAB ab49/DCSP1 labels all synaptic neuropil (*NP*) homogeneously (**A,D,G**) and presumably all synaptic boutons on muscles (**K**). By comparison with csp^{U1} null mutant preparations which were stained and processed identically (*middle column*), significant specific staining is found in retina (*R*) and brain cellular rind (*CR* in **A**) as well as in direct wing muscles (*DWM* in **K**). Examples of strong, unspecific staining are seen in fat body (**B**). MAB DCSP3 shows strong specific staining (**C, F, I, M**), albeit with a less homogeneous distribution: In the visual system, the entire lamina neuropil (*La*), three distal layers in the medulla (*Me*), four layers in the lobula plate (*LP*), and one layer in the lobula (*Lo*) stain strongly; in the central brain mushroom bodies (*MB*) and ellipsoid body (*EB*) display prominent labeling. With the highly sensitive histological protocol employed here, the remaining neuropil (**C, F, I**) as well as synaptic boutons on muscles (**M**) show weak but significant specific staining by MAB DCSP3 (*FB* fan-shaped body, *IFM* indirect flight muscles, *LPr* lateral protocerebrum, *TTM* tergotrochanter muscle). *Scale bars* 100 µm in **C** (for **A–C**) and 50 µm in **F** (for **D–M**)

the lobula complex (Fig. 6F), as well as the mushroom bodies and parts of the ellipsoid body of the central brain (Fig. 6I). With extended DAB reaction time all other neuropil regions and synaptic boutons on muscles (Fig. 6M) also display specific, albeit weak staining, indicating the presence of low concentrations of those CSP isoforms that are recognized by MAB DCSP3.

Comparison of immunolabeled sections of the mutants csp^{X1} and csp^{X2} (small deletions) with the null controls demonstrates that in these two mutants some CSP frag-

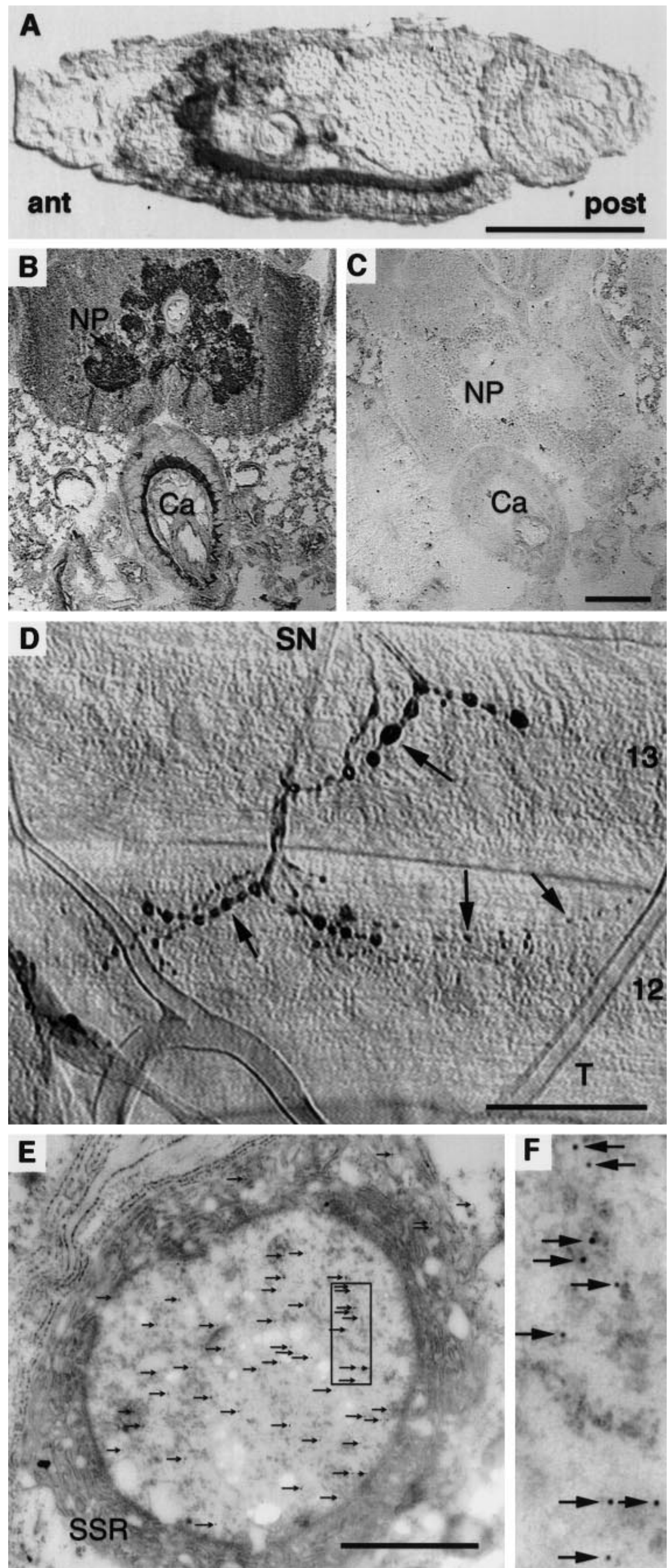


Fig. 7 Immunohistochemical staining of embryonic and larval tissues, using MAB ab49/DCSP1. **A** Sagittal frozen section from an 18-h embryo, showing early expression of CSP in the nervous system. **B, C** Sections through cardia and brain hemispheres of third instar wild-type and csp^{U1} mutant larva, demonstrating strong CSP expression in cardia (*Ca*) and neuropil (*NP*). **D** Third instar larval nerve muscle preparation (whole-mount). The antibody stains large, intermediate, and small synaptic boutons on larval body wall muscles M12 and M13 (*arrows*) (*SN* segmental nerve, *T* trachea). **E** Electron-microscopic image of an immuno-gold-labeled thin section through a synaptic bouton contacting a larval body wall muscle, verifying the predominantly presynaptic localization of CSPs in *Drosophila*. Evaluation of similar sections from 13 synapses reveal a high density of gold particles (*arrows*) in synaptic vesicle-containing regions of the boutons (*SSR* subsynaptic reticulum surrounding bouton). **F** 4.3-fold enlargement of *boxed* portion of **E**. *Scale bars* 100 μm in **A–D**, 1 μm in **E**

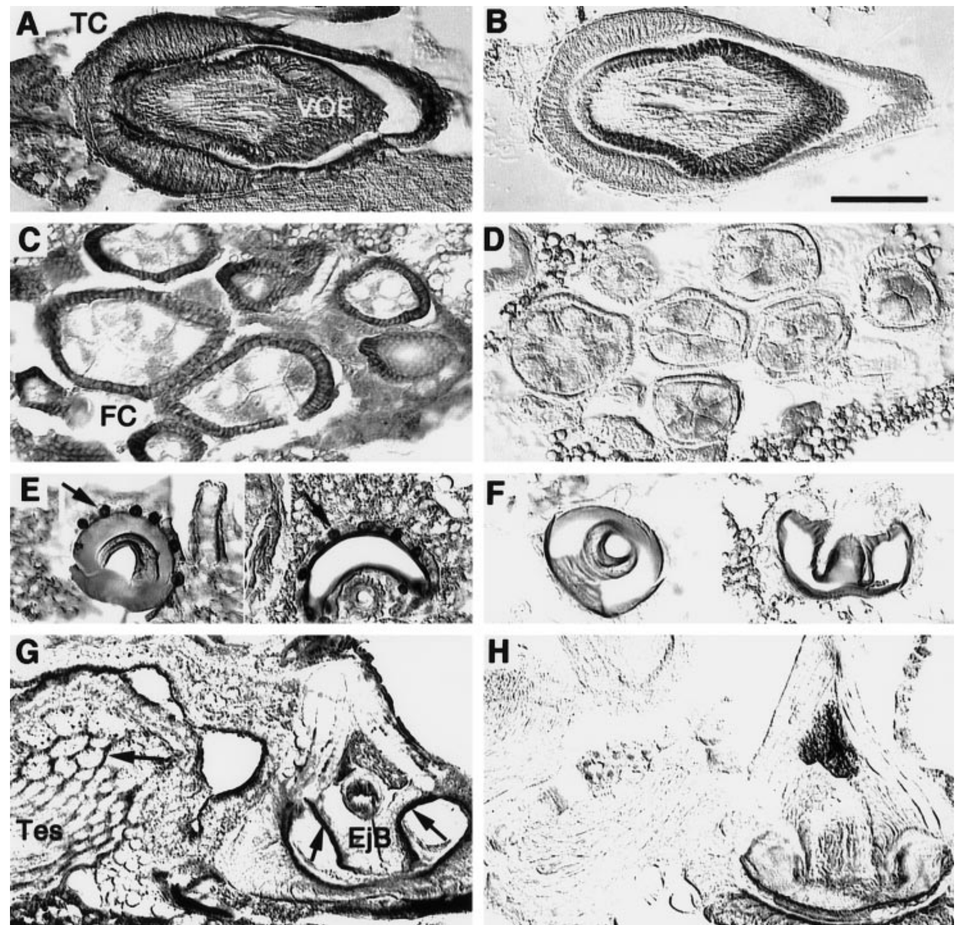


Fig. 8 Localization of CSPs in adult non-neuronal tissues employing MAB ab49/DCSP1. Strong specific staining is observed in the "tall cells" (*TC*) of the cardia (**A**), the follicle cells (*FC*) of the ovary (**C**), spherical structures of the female spermatheca (*arrows* in **E**), and certain elements of the male testis (*Tes*) and ejaculatory bulb (*EjB*) (*arrows* in **G**). The vacuolate outer epithelium (*VOE*) of the stomodeal valve and a structure of the ejaculatory bulb display strong unspecific staining in the null mutant (**B–H**). *Scale bar* 100 µm

ments are recognized by both MABs ab49/DCSP-1 and DCSP-2 in a small region of the subesophageal ganglion (data not shown, cf. "Discussion").

Embryonic and larval nervous system

Distinct immunohistochemical staining with MAB ab49/DCSP1 is seen in embryonic ganglia and ventral nerve cord as early as 10 h after egg deposition. Figure 7A shows a sagittal section through an 18 h embryo. csp^{U1} mutant embryos of approximately the same age (not shown) display only weak background labeling. In larvae, CSPs are recognized in neuropil and in the cardia (Fig. 7B, compare with csp^{U1} control in C), as well as in most or all synaptic boutons of motor neurons on the body wall muscles (Fig. 7D). Evaluation of immuno-gold-stained thin sections through these boutons demonstrates that CSPs are predominantly located in those regions of the presynaptic terminal that appear to contain synaptic vesicles (Fig. 7E,F). Note that the epitope recognized by MAB ab49/DCSP1 is destroyed by harsh fixatives, dictating glutaraldehyde-free fixation, such that ultrastructural tissue preservation is compromised and synaptic vesicles can be identified only by comparison with conventional electron-microscopic preparations (Atwood et al. 1993). High density of gold label is seen within the lumen of the boutons while only few particles are detected over the surrounding muscle tissue, including the subsynaptic reticulum. This result agrees well with the biochemical identification of CSPs as synaptic vesicle proteins (Mastrogiacomo et al. 1994; Zinsmaier et al. 1994; van de Goor et al. 1995; van de Goor and Kelly 1996) and is compatible with weak muscular CSP expression (see below).

Non-nervous tissues

For the various types of muscles in the thorax we found, in addition to the strong staining of the synaptic boutons mentioned above, also significant levels of homogeneously distributed CSPs in the contractile tissue, most prominently in the direct wing muscles (see Fig. 6K,L). The cardia, an organ of the digestive system located in the anterior thorax, shows strong accumulation of reaction product (Fig. 8A). Comparison with the mutant sections reveals, however, that most of the staining in the stomodeal valve (inner two layers of cardia) is unspecifc (Fig. 8B). Thus, in the cardia only the "tall cells" of the outer layer contain high concentrations of CSPs. Scrutinizing evaluation of our preparations suggests that the strong but unspecific staining of the inner layers visible in the mutant

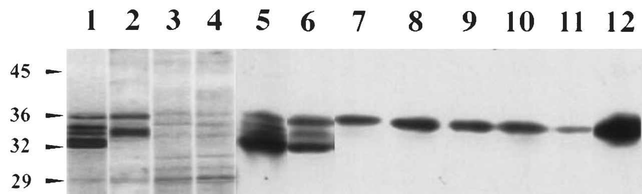


Fig. 9 Western blots of homogenized body parts or organs, stained with monoclonal antibodies against *Drosophila* CSPs. *Lanes 1–4* blot from large, high-resolution gel graphically compressed. Wild type stained with ab49/DCSP1 (1 head, *lane 1*) and DCSP3 (2 heads, *lane 2*), and mutants csp^{X1} (5 heads, *lane 3*) and csp^{U1} (5 heads, *lane 4*) stained with MAB ab49/DCSP1. Comparison with the molecular null mutant csp^{U1} (*lane 4*) reveals that the weak signals detected for the mutant csp^{X1} (*lane 3*) represent unspecific background. *Lanes 5–12* wild type stained with MAB ab49/DCSP1. One head (*lane 5*); 1 thorax (*lane 6*); 1 female abdomen (*lane 7*); 10 Malpighian tubules (*lane 8*); 20 cardia (*lane 9*); 20 ovaries (*lane 10*); 20 spermathecae (*lane 11*); 20 larval salivary glands (*lane 12*)

may be superimposed, in the wild type, on a weak homogeneous CSP expression. This may also be true for other unspecifically stained structures, e.g., salivary glands, malpighian tubules, ventriculus, intestine, and fat body (not shown). Strong specific staining is observed, however, in the follicle cells of the ovary (Fig. 8C,D), spherical structures surrounding the female spermatheca (E,F), a cell layer of the male ejaculatory bulb, and linings of the bundles of sperm tails in the testis (G,H).

Isoform analyis by Western blots

The three available monoclonal antibodies (MABs) against CSPs have been described previously (Buchner et al. 1988; Zinsmaier et al. 1990, 1994). MABs ab49/DCSP1 and DCSP2 (not shown) recognize at least four different CSP isoforms (at 32, 33, 34 and 36 kDa) in wild-type head homogenates (lane 1 of Fig. 9), while only two bands (at 33 and 36 kDa) are detected by MAB DCSP3 (lane 2). The weak signals seen with the small deletion mutant csp^{X1} (lane 3) represent unspecific background as revealed by comparison with the null mutant csp^{U1} (lane 4). Since in immunohistochemical preparations specific staining in csp^{X1} (and csp^{X2}) is observed only in a small neuropil region of the subesophageal ganglion, we assume that the amount of the corresponding CSP fragments is too small to be detected in our Western blots of head homogenates.

The immunohistochemical demonstration of the presence of CSPs in non-neuronal tissues raises the question which of the four isoforms recognizable in head homogenates is expressed in the organs of thorax and abdomen. Furthermore, due to the above-mentioned unspecific antibody cross-reactivity, it was not possible to unequivocally clarify by immunohistochemistry whether CSPs are present in several secretory organs, such as salivary glands and Malpighian tubules. Body parts such as heads, thoraces and abdomens, as well as isolated organs, were therefore separately homogenized and analyzed by Western blotting. Figure 9 demonstrates that all organs investigated [Malpighian tubules (lane 8), cardia (9), ovaries (10), spermatheca (11), larval salivary glands (12)] contain only a single CSP isoform of 36 kDa. Thus the smaller signals at 32, 33 and 34 kDa of heads, and at 32 and 33.5 kDa of thoraces, are assumed to derive from nervous system-specific CSP isoforms. Since CSPs were detected also in human blood (Coppola and Gundersen 1996), we investigated *Drosophila* hemolymph for the presence of CSPs. No Western signal was detected in larval or adult hemolymph (data not shown).

Discussion

By generating null mutants for the *csp* gene of *Drosophila* we have demonstrated that cysteine string proteins are essential for normal nervous system function. In these mutants severe neurological symptoms are observed, and paralysis at elevated temperatures is caused by disrupted excitation-secretion coupling at the presynaptic terminal (Umbach et al. 1994; Zinsmaier et al. 1994). Here we use the mutants to investigate the relevance of CSPs for dynamic aspects of synaptic transmission at permissive temperatures and to demonstrate the expression of CSPs in most or all *Drosophila* tissues. The high levels of CSPs in presynaptic terminals of neurons, the "tall" cells of the cardia, the follicle cells of the ovary, and a cell layer lining the lumen of the ejaculatory bulb may well relate to a regulated secretory function of these tissues. For other structures with high CSP concentrations, such as spermatheca or sperm bundle linings, no functional speculations seem possible at present. Wide expression of CSPs has been shown previously for vertebrates by Northern and Western analysis of isolated organs (Chamberlain and Burgoyne 1996; Chamberlain et al. 1996; Coppola and Gundersen 1996; Jacobsson and Meister 1996; Pupier et al. 1997; Zhao et al. 1997). The demonstration of CSP expression outside the nervous system in *Drosophila* makes it clear that the loss of these proteins from numerous organs may have important implications for interpreting systemic phenotypes of the mutants, such as delayed development and premature death. Several aspects of the present investigations deserve further comment.

Mutagenesis

Three variants of site-directed gene "knock-out," all exploiting the dominant eye color phenotype of the two P(w^+) insertions csp^{P1} and csp^{P2}, were used to produce deficiencies in the *csp* gene. Whereas γ-ray and "jump-out" mutagenesis involve inherently stochastic processes and generate unpredictable genetic aberrations and second site mutations, the unequal crossing-over scheme favors a precise excision of the *csp* gene. While this "clean" resection of a single gene had actually taken place in at least one of the 15 flies isolated, the scheme depends on the previous isolation of two P insertions upstream and downstream of

the gene and on an efficient mutant screening procedure, such that its general applicability is limited. Here, mass scoring for non-complementation of the temperature-sensitive paralytic phenotype of the previously obtained *csp*^{X1} allele (Eberle 1995) made a screen of 150000 chromosomes feasible.

Phenotype

The null alleles demonstrate that in the absence of intact cysteine string proteins a prolonged developmental period (Fig. 3A) can produce viable flies of severely reduced adult lifespan (Fig. 3B) which become paralyzed and die under heat stress. The lack of phenotypic differences between *csp*^{U1} (molecular null allele) and *csp*^{X1} suggests that the residual CSP fragments in the latter may be functionally irrelevant. Thus, the X-alleles also seem to represent functional null alleles. Temperature-sensitive paralysis, the most evident phenotype of the mutants, can be directly associated with a temperature-dependent breakdown of synaptic transmission. This has been analyzed independently at two different sites, the synaptic connection between adult photoreceptors and first order interneurons of the lamina, and the neuromuscular junction of the larval body wall. With the latter paradigm, it had been demonstrated that CSPs are required for presynaptic excitation-secretion coupling, while motor nerve conduction, transmitter release by spontaneous exocytosis of presynaptic vesicles, and responsivity of the muscle to externally applied transmitter remained unaffected (Umbach et al. 1994). Here, we include information on dynamic aspects of the defects as determined by quantitative evaluation of electroretinogram recordings. The data in Fig. 4 demonstrate that, at permissive temperatures, synaptic transmission in the mutants is more severely affected at high stimulus frequencies. "On" responses decrease from 72% of wild type values (at 1 Hz) to 51% (at 10 Hz) and "off" responses decrease from 30% (at 1 Hz) to near zero (at 10 Hz), except at the lowest light level at which both "on" and "off" responses in the mutant appear to recover to some extent. The small decrease in the sustained potential in the mutants is barely significant. It could indicate a function of CSPs beyond neurotransmitter release (see below) or may reflect minor variations in fly strains due to genetic background effects.

Surprisingly, the apparent reduction of peak synaptic currents in the mutants even at "permissive" temperatures, as seen both in the ERG (Fig. 4) and in larval muscle recordings (Umbach et al. 1994), does not incapacitate the nervous system of the mutants to perform all vital functions during development and after eclosion. Behavioral analysis of optomotor responses (Fig. 5) produced an even more striking result: turning responses to visual movement at threshold pattern luminance are not different from wild type. This correlates with the finding that ERG defects are smaller at low compared to high light levels. It thus appears that, near threshold, when response amplitude is limited by signal noise, CSP defects are less relevant. This might perhaps be explained by the finding that the timing of signal transmission at the synapse is affected in the mutants (Heckmann et al. 1997). Macro-patch recordings from individual synaptic boutons on larval body wall muscles at permissive temperatures (16–18°C) demonstrate that the timing of release is less precise in *csp*^{U1} mutants, leading to spread-out postsynaptic potentials of reduced amplitude, whereas the number of transmitter quanta released per stimulus is normal. This loss of temporal precision in signal transmission may perhaps be effectively masked when signals are already smeared due to photon noise at low light levels.

Tissue distribution of CSP isoforms

In small animals, immunohistochemistry represents the method of choice to determine at high resolution the distribution of the proteins expressed from the gene under study. Due to the inherent problem of antibody cross-reactivity, such data can be reliably interpreted only when a null mutant for the gene serves as a negative control. The three monoclonal antibodies employed here recognize at least two different epitopes on unmodified CSPs. Although these epitopes have not yet been mapped, it is tempting to assume that MAB DCSP3 either recognizes the 21 amino acid stretch discriminating CSP1 from CSP2 and CSP3 or the C-terminus of CSP1 and CSP2 which is different in CSP3 (cf. Fig. 1).

MABs ab49/DCSP1 and DCSP2 bind to all four CSP isoforms identifiable on Western blots of wild-type head homogenate and stain all neuropil and synaptic terminals on muscles rather homogeneously, suggesting that all synapses contain at least one of the four known isoforms. The residual CSP-specific staining pattern observed in *csp*^{X1} and *csp*^{X2} mutant brains with MAB ab49/DCSP1 and DCSP2 (data not shown) is difficult to interpret, since the abundance of the corresponding isoform(s) in the mutants is too low to give a Western signal discriminable from the unspecific background seen with *csp*^{U1} null mutants (Fig. 9). The expressed CSP fragments presumably result from the specific deletions in *csp*^{X1} and *csp*^{X2} which bring sequences of the plasmid origin (*ori*, see Fig. 2) in close proximity to CSP-coding DNA fragments. Such sequences possibly contain accidental binding sites for eukaryotic transcription factors which could bring about the observed expression patterns.

MAB DCSP3, on the other hand, binds to only two isoforms on Western blots of wild-type head homogenate. The staining pattern demonstrates cell-specific regulation of CSP expression in the brain. While isoforms recognized by MAB DCSP3 are abundant in photoreceptor terminals and synaptic neuropil of mushroom bodies and ellipsoid body in the central brain (Zinsmaier et al. 1994 and Fig. 6), their concentration in all other CSP-expressing cells and tissues is very low, and can be demonstrated only by the most sensitive detection methods.

The relationship between the three cloned cDNAs, the two mRNA signals seen in Northern blots (Zinsmaier et

al. 1994), the four protein isoforms detected by Western blots (Fig. 9), and the differential tissue staining by the available antibodies appears complex and remains to be analyzed in detail. The issue is confounded by the extensive post-translational modification that increases the apparent molecular weight of CSPs in SDS gels by several kilodaltons (van de Goor and Kelly 1996; Gundersen et al. 1996). By inference from data on *Torpedo* CSP (Gundersen et al. 1994), it is assumed that the strings of cysteines are multiply palmitoylated also in *Drosophila* CSPs, but it is unknown whether acylation is uniform with respect to the different isoforms, developmental stage, and/or cell type. Whatever the molecular mechanism, it is clear from the Western blots and the differential staining pattern of MAB DCSP3 that CSPs add to the molecular diversity of presynaptic nerve terminals in different brain regions while only a single isoform is found in other tissues. Such a nervous system-specific enhanced complexity has been emphasized previously on the basis of the fact that most known proteins involved in regulated exocytosis of vertebrates exist in several isoforms with demonstrated or possible cell-specific regulation (Südhof 1995).

CSP function

Hypotheses on the molecular role of this novel family of synaptic vesicle proteins must explain the temperature-sensitive breakdown of synaptic excitation-secretion coupling observed in the *Drosophila* mutants (Umbach et al. 1994; Zinsmaier et al. 1994). Since some DnaJ-like proteins function as molecular chaperones by interacting through the "J" domain with heat shock proteins (Hsp), it was proposed that CSPs could exert specific effects on other proteins via this domain, possibly also as chaperones (reviewed by Buchner and Gundersen 1997). Recent biochemical evidence supports this view by demonstrating that mammalian CSPs specifically activate Hsc70 ATPase, the constitutive brain Hsp70 form, and prevent aggregation of heat-denatured proteins in cooperation with Hsc70 (Braun et al. 1996; Chamberlain and Burgoyne 1997a,b). Considering the way in which the first vertebrate CSP cDNA was detected, it was attractive to speculate that in the nervous system CSPs regulate presynaptic voltage-sensitive calcium channels (Mastrogiacomo et al. 1994). This hypothesis is supported by experiments using α-latrotoxin and calcium ionophores which can trigger exocytotic transmitter release in *csp* mutants even at restrictive temperatures (Umbach and Gundersen 1997; Ranjan et al. 1998). Recently, calcium imaging demonstrated more directly that in presynaptic terminals of *csp* mutants stimulus-induced calcium entry is blocked at high temperatures (Umbach et al. 1998). Thus, in the wild-type synapse CSPs might help, and at high temperatures be required, to sensitize voltage-sensitive calcium channels, effectively restricting calcium entry into the presynaptic terminal to zones within molecular ranges of docked synaptic vesicles, where high calcium concentrations are required to trigger vesicle exocytosis. Direct biochemical evidence regarding an interaction of CSPs with the protein complexes involved in presynaptic calcium entry or exocytosis is still somewhat equivocal. While co-immunoprecipitation of synaptosomal proteins revealed no interaction of CSPs with native calcium channels (Martin-Moutot et al. 1996; Pupier et al. 1997; Leveque et al. 1998), specific binding of CSPs to a fusion protein containing a cytoplasmic segment of the α1A-subunit of P/Q-type channels has recently been reported (Leveque et al. 1998).

In *Drosophila* most of the non-neuronal cells shown here to contain CSPs, such as tall cells of cardia, ovarian follicle cells, Malpighian tubules, or salivary glands, are known to perform major secretory function. This could indicate that CSPs are primarily involved secretory mechanisms. However, the presence of CSPs in non-secretory tissues like muscles raises the possibility that CSPs may act as chaperones with different targets and different functions in different cell types. Although at present no information on a specific function of CSPs in non-neuronal tissues of *Drosophila* is available, the mutants now make such questions amenable to experimental analysis.

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References

Ashburner M (1989a) *Drosophila*. A laboratory handbook. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Ashburner M (1989b) *Drosophila*. A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Atwood HL, Govind CK, Wu CF (1993) Differential ultrastructure of synaptic terminals on ventral longitudinal abdominal muscles in *Drosophila* larvae. J Neurobiol 23:1008–1024

Ballinger DG, Benzer S (1989) Targeted gene mutations in *Drosophila*. Proc Natl Acad Sci USA 86:9402–9406

Bier E, Vaessin H, Sheperd S, Lee K, McCall K, Barbel S, Ackerman L, Carretto R, Uemura T, Grell E, Jan LY, Yan YN (1989) Searching for pattern and mutation in the *Drosophila* genome with a P-lacZ vector. Genes Dev 3:1273–1287

Braun JEA, Scheller RH (1995) Cysteine string protein, a DnaJ family member, is present on diverse secretory vesicles. Neuropharmacology 34:1361–1369

Braun JEA, Wilbanks SM, Scheller RH (1996) The cysteine string secretory vesicle protein activates Hsc70 ATPase. J Biol Chem 271:25989–25993

Buchner E, Gundersen CB (1997) The DnaJ-like cysteine string protein and exocytotic neurotransmitter release. Trends Neurosci 20:223–227

Buchner E, Buchner S, Crawford G, Mason WT, Salvaterra PM, Sattelle DB (1986) Choline acetyltransferase-like immunoreactivity in the brain of *Drosophila melanogaster*. Cell Tissue Res 246:57–62

Buchner E, Bader R, Buchner S, Cox J, Emson PC, Flory E, Heizmann CW, Hemm S, Hofbauer A, Oertel WH (1988) Cell-specific immuno-probes for the brain of normal and mutant *Drosophila melanogaster*. Cell Tissue Res 253:357–370

Chamberlain LH, Burgoyne RD (1996) Identification of a novel cysteine string protein variant and expression of cysteine string proteins in non-neuronal cells. J Biol Chem 271:7320–7323
Chamberlain LH, Burgoyne RD (1997a) Activation of the ATPase activity of heat-shock proteins Hsc70/Hsp70 by cysteine-string protein. Biochem J 322:853–858
Chamberlain LH, Burgoyne RD (1997b) The molecular chaperone function of the secretory vesicle cysteine string proteins. J Biol Chem 272:31420–31426
Chamberlain LH, Henry J, Burgoyne RD (1996) Cysteine string proteins are associated with chromaffin granules. J Biol Chem 271:19514–19517
Coppola T, Gundersen C (1996) Widespread expression of human cysteine string proteins. FEBS Lett 391:269–272
Eberle KK (1995) Mutagenese und Funktionsanalyse des *csp*-Gens von *Drosophila melanogaster*. PhD thesis, Universität Würzburg
Goor J van de, Kelly R (1996) Association of *Drosophila* cysteine string proteins with membranes. FEBS Lett 380:251–256
Goor J van de, Ramaswami M, Kelly R (1995) Redistribution of synaptic vesicles and their proteins in temperature-sensitive $shibire^{ts1}$ mutant *Drosophila*. Proc Natl Acad Sci USA 92:5739–5743
Grell RF (1978) A comparison of heat and interchromosomal effects on recombination and interference in *Drosophila melanogaster*. Genetics 89:65–77
Gundersen CB, Umbach JA (1992) Suppression cloning of the cDNA for a candidate subunit of a presynaptic calcium channel. Neuron 9:527–537
Gundersen CB, Mastrogiacomo A, Faull K, Umbach JA (1994) Extensive lipidation of a *Torpedo* cysteine string protein. J Biol Chem 269:19197–19199
Gundersen CB, Umbach JA, Mastrogiacomo A (1996) Cysteine-string proteins: a cycle of acylation and deacylation? Life Sci 58:2037–2040
Guo Y, Gillan A, Török T, Kiss I, Dow JAT, Kaiser K (1996) Site-selected mutagenesis of the second chromosome via plasmid rescue of lethal P-element insertions. Genome Res 6:972–979
Hamilton BA, Palazzolo JM, Chang JH, Raghavan KV, Mayeda CA, Whittney MA, Meyerowitz EM (1991) Large scale screen for transposon insertions into cloned genes. Proc Natl Acad Sci USA 88:2731–2735
Harlow E, Lane D (1989) Antibodies: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
Heckmann M, Adelsberger H, Dudel J (1997) Evoked transmitter release at neuromuscular junctions in wild type and cysteine string protein null mutant larvae of *Drosophila*. Neurosci Lett 228:167–170
Heisenberg M (1971) Separation of receptor and lamina potentials in the electroretinogram of normal and mutant *Drosophila*. J Exp Biol 55:85–100
Heisenberg M, Götz KG (1975) The use of mutations for the partial degradation of vision in *Drosophila melanogaster*. J Comp Physiol 98:217–241
Hofbauer A (1991) Eine Bibliothek monoklonaler Antikörper gegen das Gehirn von *Drosophila melanogaster*. Habilitationsschrift, Universität Würzburg
Jacobsson G, Meister B (1996) Molecular components of the exocytotic machinery in the rat pituitary gland. Endocrinology 137:5344–5356
Kaiser K, Goodwin SF (1990) "Site-selected" transposon mutagenesis of *Drosophila*. Proc Natl Acad Sci USA 87:1686–1690
Kohan SA, Pescator M, Brecha NC, Mastrogiacomo A, Umbach JA, Gundersen CB (1995) Cysteine string protein immunoreactivity in the nervous system and adrenal gland of rat. J Neurosci 15:6230–6238
Leveque C, Pupier S, Marqueze B, Geslin L, Kataoka M, Takahashi M, De Waard M, Seagar M (1998) Interaction of cysteine string proteins with the alpha1A subunit of the P/Q-type calcium channel. J Biol Chem 273:13488–13492
Lindsley DL, Zimm G (1992) The genome of *Drosophila melanogaster*. Academic Press, San Diego
Martin-Moutot N, Charvin N, Leveque C, Sato K, Nishiki T, Takahashi M, Seagar M (1996) Interaction of SNARE complexes with P/Q-type calcium channels in rat cerebellar synaptosomes. J Biol Chem 271:6567–6570
Mastrogiacomo A, Gundersen CB (1995) The nucleotide and deduced amino acid sequence of a rat cysteine string protein. Mol Brain Res 28:12–18
Mastrogiacomo A, Parsons MS, Zampighi GA, Jenden DJ, Umbach JA, Gundersen CB (1994) Cysteine string proteins: a potential link between synaptic vesicles and presynaptic Ca^{2+} channels. Science 263:981–982
Melzig J, Buchner S, Wiebel J, Wolf R, Burg R, Pak WL, Buchner E (1996) Genetic depletion of histamine from the nervous system of *Drosophila* eliminates specific visual and mechanosensory behavior. J Comp Physiol [A] 179:763–773
Pak WL (1975) Mutants affecting the vision of *Drosophila melanogaster*. In: King RC (ed) Handbook of genetics. Plenum Press, New York, pp 703–733
Pupier S, Leveque C, Marqueze B, Kataoka M, Takahashi M, Seagar MJ (1997) Cysteine string proteins associated with secretory granules of the rat neurohypophysis. J Neurosci 17:2722–2727
Rafti F, Scarvelis D, Lasko PF (1996) A *Drosophila melanogaster* homologue of the human DEAD-box gene *DDX1*. Gene 171:225–229
Ranjan R, Bronk P, Zinsmaier KE (1998) Cysteine string protein is required for calcium secretion coupling of evoked neurotransmission in *Drosophila* but not for vesicle recycling. J Neurosci 18:956–964
Sambrook J, Fritsch FE, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
Silver PM, Way FC (1993) Eukaryotic DnaJ homologs and the specificity of Hsp70 activity. Cell 74:5–6
Südhof TC (1995) The synaptic vesicle cycle: a cascade of protein-protein interactions. Nature 375:645–653
Umbach JA, Gundersen CB (1997) Evidence that cysteine string proteins regulate an early step in the Ca^{2+}-dependent secretion of neurotransmitter at *Drosophila* neuromuscular junctions. J Neurosci 17:7203–7209
Umbach JA, Zinsmaier KE, Eberle KK, Buchner E, Benzer S, Gundersen CB (1994) Presynaptic dysfunction in *Drosophila csp* mutants. Neuron 18:899–907
Umbach JA, Saitoe M, Kidokoro Y, Gundersen CB (1998) Attenuated influx of calcium ions at nerve endings of *csp* and *shibire* mutant *Drosophila*. J Neurosci 18:3233–3240
Walter N (1992) (I) Isolation and characterization of new *Drosophila* tyrosine kinase genes and (II) the development of a new method to introduce mutations in cloned genes. PhD thesis, University of Zürich
Welch WJ, Gambetti P (1998) Chaperoning brain diseases. Nature 392:23–24
Zhao C-M, Jacobsson G, Chen D, Håkanson R, Meister B (1997) Exocytotic proteins in enterochromaffin-like (ECL) cells of the rat stomach. Cell Tissue Res 290:539–551
Zinsmaier KE, Hofbauer A, Heimbeck G, Pflugfelder GO, Buchner S, Buchner E (1990) A cysteine-string protein is expressed in retina and brain of *Drosophila*. J Neurogenetics 7:15–29
Zinsmaier KE, Eberle KK, Buchner E, Walter N, Benzer S (1994) Paralysis and early death in cysteine string protein mutants of *Drosophila*. Science 263:977–980