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Impairment of central pattern generation in *Drosophila* cysteine string protein mutants

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Abstract The cysteine string proteins are integral synaptic vesicle proteins, critical for fast, Ca^{2+}-regulated exocytosis. *Drosophila* larvae with null mutations for the cysteine string protein (*csp*) gene have temperature-sensitive impairments of neurotransmission and presynaptic calcium removal at the neuromuscular junction. Using the larval *Drosophila* preparation to examine central pattern generation, we characterized the temperature sensitivity of locomotor patterns in wildtype and *csp* mutant larvae. Intraburst frequency of motoneuronal activity reached 100 Hz and was sufficiently high to rescue the temperature-sensitive synaptic failure in the mutant. Nevertheless, we show that deletion of the *csp* gene resulted in a severe deficiency in the generation of coordinated larval motor rhythms. *Csp* mutants that could generate patterned motor activity had slower, poorly coordinated rhythms with altered temperature sensitivity. We conclude that the temperature sensitive paralysis characteristic of *csp* mutants is not a direct result of synaptic failure at neuromuscular junctions, as might be expected, but is the result of a failure of locomotor circuit operation at a higher integrative level.

Keywords Cysteine string proteins · Central pattern generation · *Drosophila* · Temperature · Synaptic transmission

Introduction

Fast, excitation-coupled release of neurotransmitter, mediated by synaptic vesicle fusion with the presynaptic membrane, is a highly regulated physiological mechanism. Cysteine string protein (CSP) is a synaptic vesicle protein first described in *Drosophila* mutants showing temperature-sensitive paralysis (Zinsmaier et al. 1994). CSP has been identified as a significant component regulating vesicle exocytosis (Umbach and Gundersen 1997; Ranjan et al. 1998; Chamberlain and Burgoyne 2000; Graham and Burgoyne 2000); however, its exact mechanism of action remains elusive. CSP was originally suggested to increase presynaptic Ca^{2+} channel activity (Mastrogiacomo et al. 1994; Umbach et al. 1998). Recent work has shown that CSP does not affect Ca^{2+} channel currents of PC-12 cells (Chamberlain and Burgoyne 1998), insulin-secreting cells (Brown et al. 1998) and *Drosophila* neuromuscular junctions (Morales et al. 1999). *Drosophila* null mutants exhibit larger stimulus-evoked Ca^{2+} signals at neuromuscular junctions despite a reduction in neurotransmitter release (Dawson-Scully et al. 2000), suggesting CSP may act downstream of Ca^{2+} entry possibly by enhancing the Ca^{2+} sensitivity of exocytosis or by stabilizing Ca^{2+} extrusion from the presynaptic active zone. Research on the physiological role of CSP to date has been undertaken on neuromuscular junctions (Umbach et al. 1994, 1998; Umbach and Gundersen 1997; Ranjan et al. 1998; Dawson-Scully et al. 2000) and several nonsynaptic models for vesicular release (Brown et al. 1998; Chamberlain and Burgoyne 1998; Zhang et al. 1998; Graham and Burgoyne 2000), but it cannot be tacitly assumed that its role at central synapses is identical. To address this question, we have adapted the *Drosophila* larval preparation to permit the investigation of motor rhythms generated by the central nervous system (CNS).

Drosophila is ideal for molecular investigations of neural function due to its amenability to genetic manipulation. The larval preparation is widely used as a model system for investigating synaptic physiology at the neuromuscular junction (Keshishian et al. 1996); however, there have been no investigations of central synaptic physiology in larvae, and very few in the adult (Trimarchi and Murphey 1997). Centrally generated

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locomotor rhythms provide a convenient assay for normal or abnormal function of CNS synapses (Marder and Calabrese 1996; Calabrese 1998). Although genetic factors influencing locomotor behaviour have been investigated (Osborne et al. 1997; Wang et al. 1997), there have been few investigations of centrally-generated rhythmic activity in the *Drosophila* larva (Budnik et al. 1990; Gorczyca et al. 1991; Cattaert and Birman 2001) and the underlying peristaltic locomotor patterns have yet to be fully described. Until recently, no consistent, stable preparation for examining such circuit function in this organism has been available. Here, we show that locomotor rhythms generated in the *Drosophila* larval CNS of *csp* mutants are slower and uncoordinated in comparison with wildtype, supporting the conclusion that CSP functions physiologically at central synapses involved in endogenous central pattern generation. Additionally, we conclude that temperature-sensitive paralysis of the *csp* mutant is not a result of neuromuscular transmission failure and suggest that it is a result of failure of locomotor circuit function due to alterations in the dynamics of central synaptic transmission.

Materials and methods

Fly stocks

Flies were grown on standard cornmeal medium at 25°C at 60–70% relative humidity. Physiological comparisons were made between wandering third-instar larvae of the Canton-S strain and two *csp* mutant lines supplied by K.E. Zinsmaier. The homozygous semi-lethal csp^{U1} mutant was obtained by heat shock-enhanced recombination between two P elements flanking the *csp* gene producing complete deletion of the *csp* gene (Eberle et al. 1998). The csp^{X1} mutant, generated by P element jump-out mutagenesis, lacks the *csp* promoter and the first exon containing the translational start site (Zinsmaier et al. 1994). Both *csp* mutants are phenotypically indistinguishable (Eberle et al. 1998; Dawson-Scully et al. 2000). *Csp* rescue flylines were constructed using a genomic DNA fragment to rescue the mutant phenotype (Zinsmaier et al. 1994). Mutations were maintained with a TM6 Balancer chromosome in a *white* genetic background. The dominant *Tubby* mutation of TM6 was used to identify genotypes where *csp* homozygous larvae were of the non-*Tubby* phenotype. The dataset was obtained from 36 wildtype (Canton-S), 12 csp^{U1}, 6 csp^{X1} and 9 *csp* rescue preparations. As there were no significant differences between the mutant flylines in success rate for motor pattern generation or rhythm frequency ($P > 0.05$), results from both mutants were combined to increase the size of the mutant dataset.

Electrophysiology

Wandering third-instar larvae were dissected in Schneider's cell culture medium (Gibco) to reveal the nervous system and body-wall muscles, as previously described (Jan and Jan 1976). All electrophysiological experiments were conducted in standard haemolymph-like solution (HL3; Stewart et al. 1994) with 0.8 mmol l^{-1} Ca^{2+}. Temperature was held at individual discrete points (20°C, 25°C, 30°C and 35°C) for 10–20 min before increasing to the next level. Preparation temperature was controlled with a Nichrome heating coil around an inlet pipette leading from a reservoir held at room temperature. Temperature was monitored with a copper/constantan thermocouple (0.2 mm; BAT-12, Sensortek, Clifton, N.J.) placed adjacent to the larval head.

For experiments with the CNS removed, motor neuron axons were stimulated with just-suprathreshold square wave pulses (0.3 ms duration) using a suction electrode on the severed end of the innervating abdominal nerve (Kurdyak et al. 1994). After failure of synaptic transmission in *csp* mutants at nonpermissive temperatures, increasing stimulus strength did not restore transmission and we are confident that the results described were not due to a failure to stimulate the axon effectively (see also Dawson-Scully et al. 2000). For recordings of endogenous bursts of motor activity, the CNS was left intact (Fig. 1). Dual intracellular recordings were made with glass microelectrodes (filled with 3 mol l^{-1} KAc) from abdominal muscle 6 of segments 3 and 5. Penetrations were made simultaneously on the left (segment 3) and right (segment 5) of the larval body midline. Abdominal muscle 6 was selected as both its size and location would implicate it as contributing to the peristaltic locomotion of *Drosophila* larvae. Previous recordings of motor activity have been made extracellularly by touching the muscle fibres with a glass suction electrode (no suction applied) (Budnik et al. 1990). However, we found that intracellular recordings of fictive motor patterns, monitored peripherally in the larval abdominal muscles, provided an increased preparation stability, which was essential to maintain recording from two muscles simultaneously during inevitable muscle contractions.

A similar electrophysiological approach for recording *Drosophila* motor patterns has been recently developed independently, but using single microelectrode penetrations of muscle 6 (Cattaert and Birman 2001). Our dual-electrode arrangement permits a precise characterization of the locomotor pattern phasing between serial segments of the same muscle. Intracellular recordings were amplified using an Axoclamp-2A amplifier (Axon Instruments, Foster City, Calif.) in bridge mode and recorded with the MacLab/4S data acquisition system (AD Instruments). Significant differences ($P < 0.05$) between flylines were assessed using unpaired

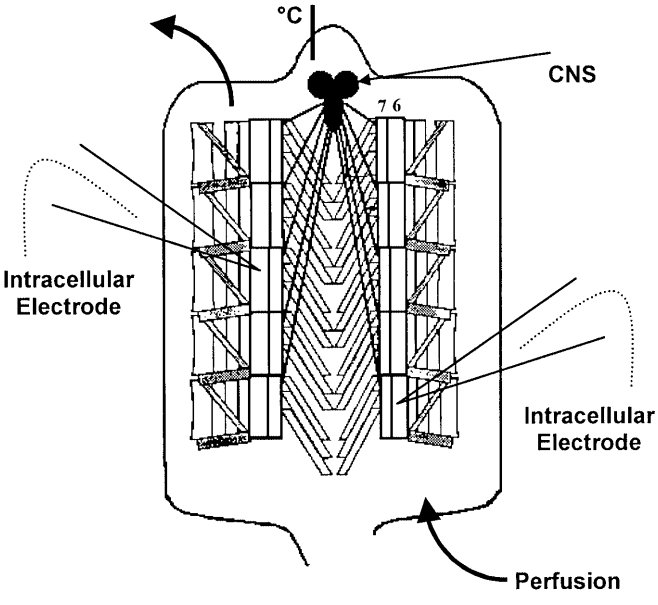

Fig. 1 Experimental arrangement for recording larval *Drosophila* motor patterns. The standard preparation for investigations of synaptic physiology at the neuromuscular junction (Jan and Jan 1976) was adapted for recording fictive motor patterns generated by the central nervous system (CNS). The larva was pinned and dissected, leaving the CNS and motor connections intact. Dual intracellular recordings were made simultaneously from ventral abdominal muscle 6 of segments 3 and 5 on the left and right sides of the body midline, respectively. Temperature-controlled saline was perfused over the preparation from posterior to anterior and the temperature (°C) was monitored with a thermocouple placed adjacent to the larval head

t-test or its nonparametric counterpart, Mann-Whitney rank sum test (motor rhythm frequency and phasing) and χ^2-test (motor pattern success).

Results

Central pattern generation in larval *Drosophila*

Abdominal muscle 6 of *Drosophila* is innervated by two identified centrally located motor neurons which differ in their synaptic physiology, producing different sized excitatory junction potential (EJP) amplitudes (Keshishian et al. 1996). In this muscle, spontaneous bursts of activity from the CNS were evident, giving rise to two characteristic sizes of EJPs (Fig. 2a). Instantaneous intraburst frequency for both motor axons ranged between 50 Hz and 80 Hz, and often reached values higher than 100 Hz. Dual intracellular recordings from ventral abdominal muscle 6 in segments 3 and 5 on opposite sides of the midline demonstrated rhythmic activity coordinated between the left and right sides of the body and synchronized with an appropriate phase delay between serial muscle segments, constituting a coherent motor pattern (Marder and Calabrese 1996; Skinner and Mulloney 1998). Larval abdominal muscles received stable, long-lasting rhythmical bursts of neural input from the CNS (Fig. 2b). At 25°C, the rhythmic activity was coordinated in abdominal muscle 6 with a characteristic phase delay between segments 3 and 5 (Fig. 2c) corresponding to anterior to posterior waves of muscular contraction as occurs during retrograde peristaltic locomotion. The rhythmical bursts of activity were coordinated between the left and right sides of the body, as shown by the synchronicity of bursting activity when recording from muscle 6 in the same segment, but on the opposite side of the midline (Fig. 2d).

A reverse coordination of rhythmic bursting (i.e. posterior to anterior waves of contraction as in forward locomotion) has also been described recently (Cattaert and Birman 2001). We recorded this type of motor pattern but less frequently (11%). Therefore we have restricted our analysis to the more prevalent type of coordinated rhythmic activity. Endogenous backward-coordinated motor sequences occurred spontaneously at 20°C in 44% of wildtype preparations ($n = 36$). Increasing the temperature of the superfusing saline in sequential 5°C steps however, reliably evoked motor sequences in previously silent preparations, such that by 30°C there was a 97% success rate for CNS-generated motor patterns for all wildtype preparations. Increasing the temperature increased the frequency of locomotor rhythms (Fig. 3). In the example presented, rhythm frequency increased from 0.13 Hz at 25°C to 0.20 Hz at 30°C and eventually 0.38 Hz at 35°C. At the upper range of recorded temperatures, the bursting activity became noticeably less coordinated than at lower temperatures.

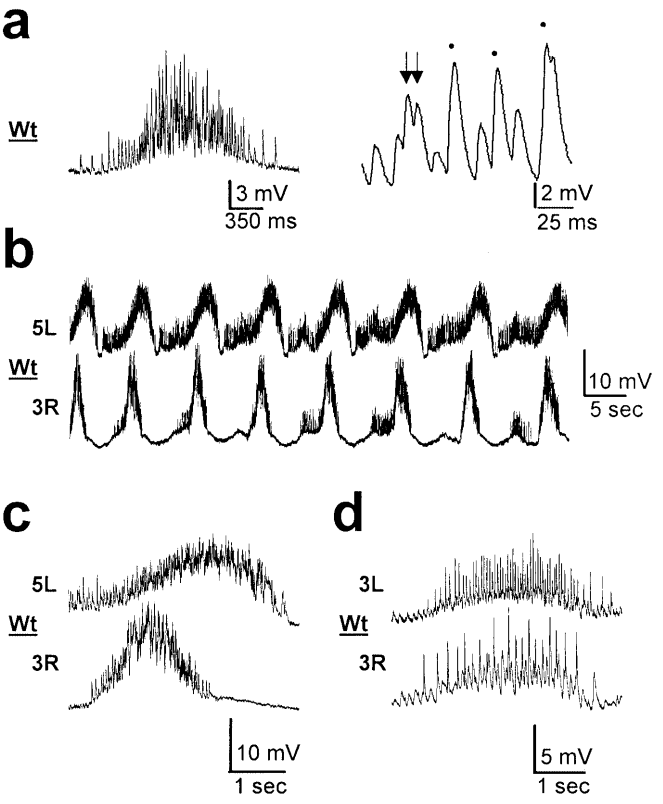

Fig. 2a–d Coordinated patterns of electrical activity of wildtype larval *Drosophila* CNS. **a** Endogenous burst of electrical activity from a wildtype preparation, recorded intracellularly from muscle 6, segment 3 at 25°C (*left*). The same trace expanded (*right*) demonstrates input is from two motor axons, each with a characteristic excitatory junction potential (EJP) amplitude. The *filled circles* indicate the larger amplitude EJP. *Arrows* indicate two EJPs, separated by 10 ms, giving an instantaneous intraburst firing frequency of 100 Hz. **b** Long-lasting stable rhythmical bursts of activity can be reliably recorded intracellularly from segments 3 and 5 of abdominal muscle 6 at 25°C. These bursts are peripherally recorded, but reflect the activity generated via central circuits. In each panel, *L* refers to recording from muscle 6 left of the body midline and *R* refers to recording right of the body midline. Note that, in this and subsequent figures, there is some movement-induced artifact associated with the contraction of surrounding body wall muscles, but that most of the depolarization associated with each burst can be attributed to the summation of EJPs in high-frequency bursts. **c** Individual bursts within the rhythm were coordinated down the larval body, with muscle segment 5 receiving input from the CNS following muscle segment 3, with a characteristic phase delay. This trace is an enlargement of the first burst in **b**. **d** Burst activity was coordinated across the body midline as well. This is demonstrated by an absence of phase difference between individual bursts recorded simultaneously from muscle 6 in segment 3 on both sides of the larval body at 25°C. Small events are attributable to electrical connections with adjacent active muscles

Rescue of synaptic transmission in csp null mutants at physiologically relevant frequencies

Temperature-sensitive disruption of neuromuscular transmission in *Drosophila* is characteristic of *csp* null mutants exposed to temperatures above 29°C for 10–15 min (Chamberlain and Burgoyne 2000). Typically,

EJPs evoked by low-frequency stimulation disappear entirely at nonpermissive temperatures (Umbach et al. 1994); however, it has been demonstrated that the synaptic defect can be partially rescued with high-frequency stimulation (Dawson-Scully et al. 2000). Recording from *csp* null mutants revealed that spontaneous CNS-generated bursts of activity could occur at nonpermissive temperatures (e.g. 35°C, Fig. 4a). The two motor axons produced impulses at intraburst frequencies equivalent to wildtype and sufficient to restore synaptic transmission. In *csp* larval preparations with the CNS removed, EJP amplitudes evoked by direct low-frequency stimulation of both motor axons were reduced in comparison to controls at room temperature and were completely absent at 32°C (Fig. 4b). However, recording from the same preparations at a nonpermissive temperature showed that high stimulation frequencies could rescue the temperature-induced disruption of synaptic transmission (Fig. 4c). *Csp* mutants lack successful synaptic transmission during the initial stimuli of a high-frequency train (Dawson-Scully et al. 2000). In the example presented here (Fig. 4c), the first stimulus in the train was ineffective to evoke an EJP. After the second stimulus an EJP was evident and over approximately 50 ms successive EJPs facilitated to reach an amplitude comparable to the initial wildtype EJP. Thus, at centrally generated frequencies, *csp* null mutants exhibit bursts of EJPs, even at elevated temperatures. However, the variable success rate of synaptic transmission (Dawson-Scully et al. 2000) and delay to reach appreciable amplitude (Fig. 4c) during the initial phase of high-frequency stimulation, if present at central synapses, would be expected to contribute to abnormal burst timing (Marder 1998). We thus predicted that the synaptic defects in *csp* null mutants would pose a major barrier for coordinated central pattern generation and locomotion.

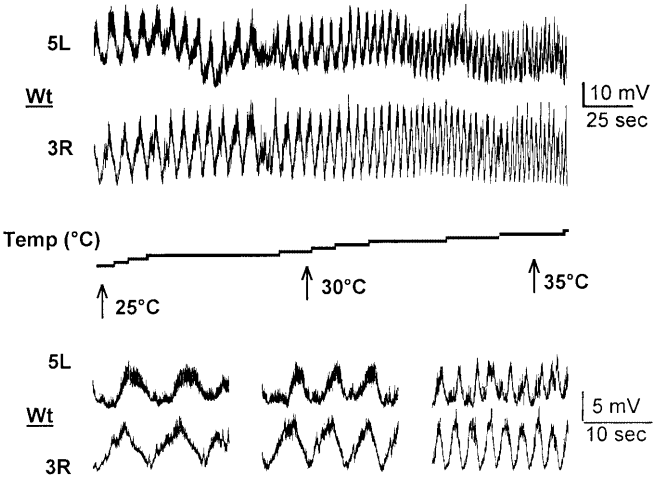

Fig. 3 Increasing the temperature increased frequency of locomotor rhythms in *Drosophila* larvae. In the example presented (*top trace*), a continuous recording of 5 min duration was made concurrently from muscle 6 of segments 3 and 5, from 25°C to 35°C. At each corresponding temperature point a 20-s section has been expanded (*bottom traces*), illustrating the increase in rhythm frequency with increasing temperature

Temperature dependency of motor rhythms in *csp* null mutants and wildtype

We compared the temperature sensitivity of locomotor patterns generated by the larval CNS in wildtype and *csp*

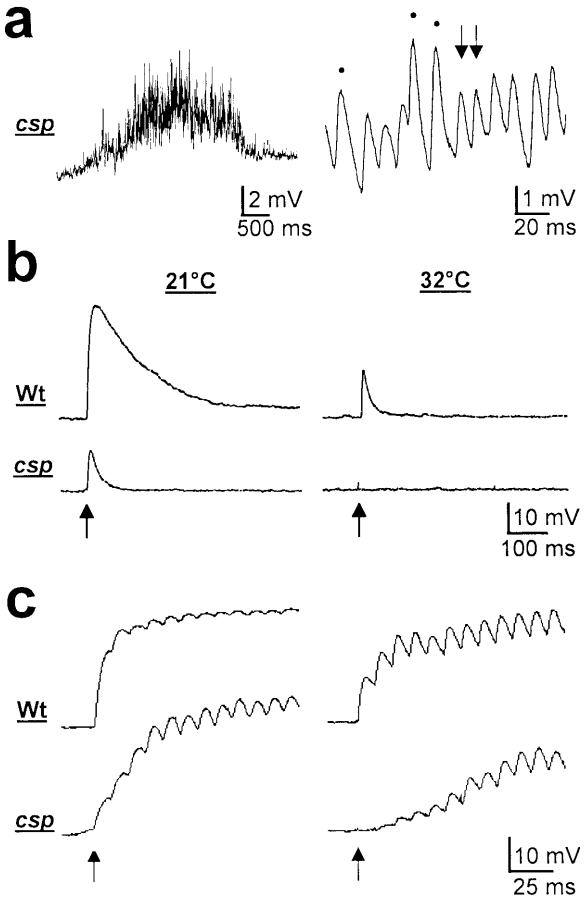

Fig. 4a–c Physiological characterization of the *csp* mutant phenotype. **a** Endogenous bursts of electrical activity are evident at 35°C in *csp* null mutant *Drosophila* larvae, recorded intracellularly from muscle 6, segment 3 (*left*). The same trace expanded (*right*) confirms that muscle 6 in the mutant also receives input from two motor axons with two characteristic EJP amplitudes. The *filled circles* indicate the larger amplitude EJP. *Arrows* indicate two EJPs, separated by 10 ms, giving an instantaneous intraburst firing frequency of 100 Hz. **b** Comparison of wildtype (*Wt*) and cysteine string protein (*csp*) null mutants demonstrates that EJPs evoked by nerve stimulation were reduced at room temperature (21°C) in mutant larvae. Exposure to elevated temperatures (32°C) completely blocked synaptic transmission at low-frequency stimulation in *csp* mutants. *Arrows* in this panel indicate stimulation. **c** The temperature-sensitive block of synaptic transmission was partially rescued in *csp* mutants during nerve evoked stimulation at 100 Hz, a frequency naturally produced by the larval CNS. At 21°C, *csp* mutant EJP amplitudes were smaller and demonstrated increased facilitation. At 32°C, where EJPs are blocked in *csp* mutants at low frequency, stimulation at 100 Hz restored synaptic transmission. The *arrows* in this panel indicate the start of stimulation train at 100 Hz

null mutants. The ability of the CNS to generate motor rhythms at any temperature was compromised by the *csp* mutation. Wildtype preparations had a 97% success rate for exhibiting a motor sequence at any temperature (Fig. 5). In *csp* null mutants however, the percentage of preparations exhibiting a motor sequence at any temperature was reduced by 50% in comparison with wildtype (Wildtype versus *csp*: $\chi^2 = 17.76$, $df = 3$, $P < 0.01$). The wildtype success rate for motor pattern generation was restored in *csp* rescue preparations (Wildtype versus *csp* rescue: $\chi^2 = 1.18$, $df = 3$, $P > 0.5$). In wildtype *Drosophila*, the frequency of rhythmical bursting in muscle 6 increased with temperature; however, intersegmental phasing was unaffected by temperature (Fig. 6a). In some successful preparations, rhythmic bursting activity in *csp* null mutants could be appropriately coordinated across the body midline and phased between segments; however, the rhythm frequency was markedly slower than in wildtype (Fig. 6b). As temperature increased from 20–35°C, rhythmic activity and pattern phasing of *csp* mutant preparations became increasingly erratic, although random bursts of motor activity were still evident at temperatures nonpermissive for low-frequency neuromuscular transmission.

Frequency and coordination of motor rhythms

Statistical comparison of wildtype and *csp* larvae confirmed that motor rhythms were indeed reduced in frequency for the mutants (Fig. 7a). Furthermore, the rate at which rhythm frequency changed with temperature was most affected in the mutant at low temperatures. Between 25–30°C, Q_{10} values for rhythm frequency in wildtype and *csp* were identical (Wildtype = 3.27 ± 0.38; *csp* = 3.24 ± 2.17; $t = 0.37$, $df = 19$, $P = 0.71$); however, between 20°C and 25°C Q_{10} was much reduced in the mutants (Wildtype = 5.32 ± 1.27 >> *csp* = 0.96 ± 0.21; $t = 2.26$, $df = 14$, $P = 0.04$). This result is consistent with previously characterized defects of adult *csp* null mutants at permissive temperatures. The mutants have been described as having a slow, uncoordinated locomotor phenotype (Zinsmaier et al. 1994) and a reduced optomotor response (Eberle et al. 1998). Restoration of the *csp* gene (*csp* rescue line) completely rescued the CNS motor pattern, emphasizing that the deficiency can be directly linked to the gene deletion (Fig. 7a). We compared the phasing of motor

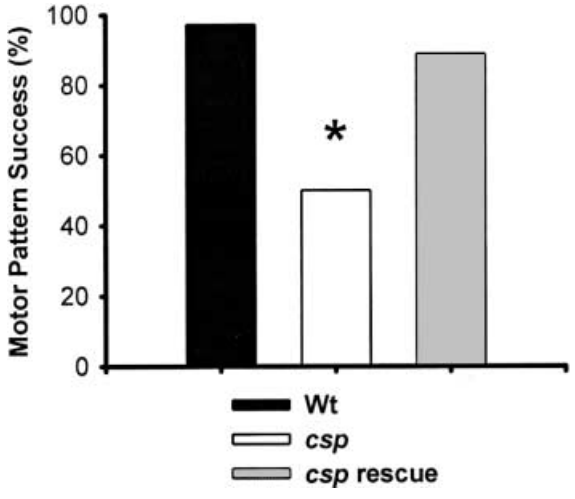

Fig. 5 Impaiment of centrally generated motor patterns in *csp* null mutants. The success rate for central pattern generation was reduced in *csp* preparations in comparison to wildtypes. The wildtype success rate for motor pattern generation was restored in *csp* rescue preparations, where the *csp* gene has been reinserted into the genome. The results were obtained from 36 wildtype, 18 *csp* and 9 *csp* rescue preparations

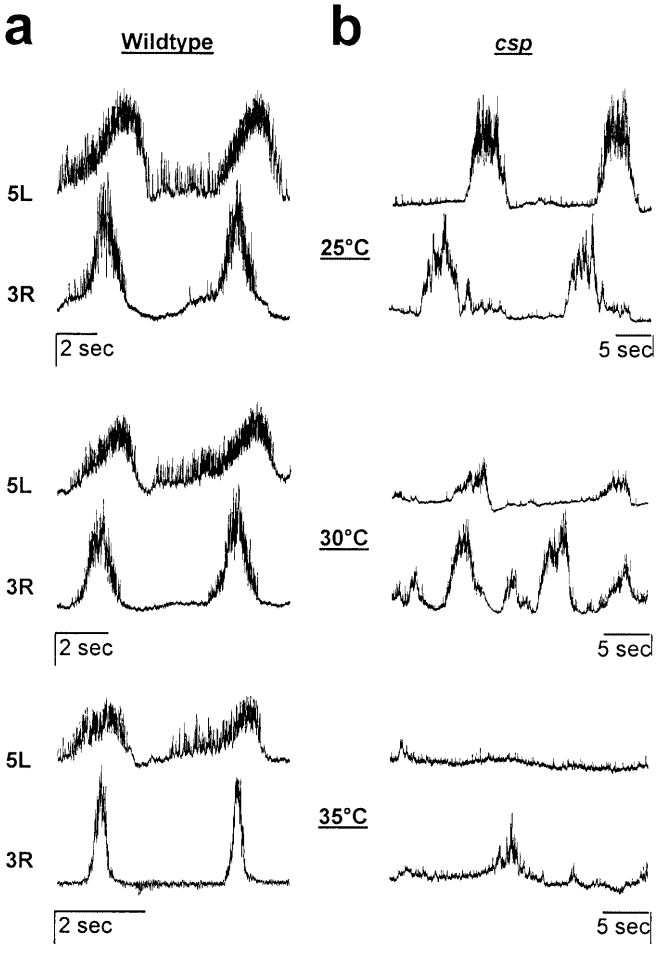

Fig. 6a,b Altered temperature sensitivity of rhythmic activity of the larval CNS in *Drosophila csp* mutants. **a** Rhythmical bursts from wildtype *Drosophila* demonstrate that as temperature was increased, the frequency of bursts increased while phase delay between bursts was largely unaffected. Intracellular recordings were made simultaneously from muscle 6, segments 5 (*L*, left of body midline) and 3 (*R*, right of body midline). Note that each 2-s burst trace has been normalized for period (timescale increases with temperature) to emphasize phase constancy with increased temperature. *Vertical scalebars* in **a** and **b** are each 5 mV. **b** Frequency of rhythmical bursts in *csp* mutants were significantly slower than in wildtype flies at temperatures above 20°C. Increasing ambient temperature caused irregularity in the bursting pattern, although not a complete absence of bursting activity

patterns in wildtype and mutants, determined from the peaks of the burst in segment 5 and segment 3, and expressed as a fraction of total cycle duration. *Csp* null mutant preparations demonstrated a marked and statistically significant increase in the variance of pattern phasing ($P < 0.01$), although comparison between the means of wildtype, *csp,* and *csp* rescue larvae did not reveal any significant difference (Fig. 7b).

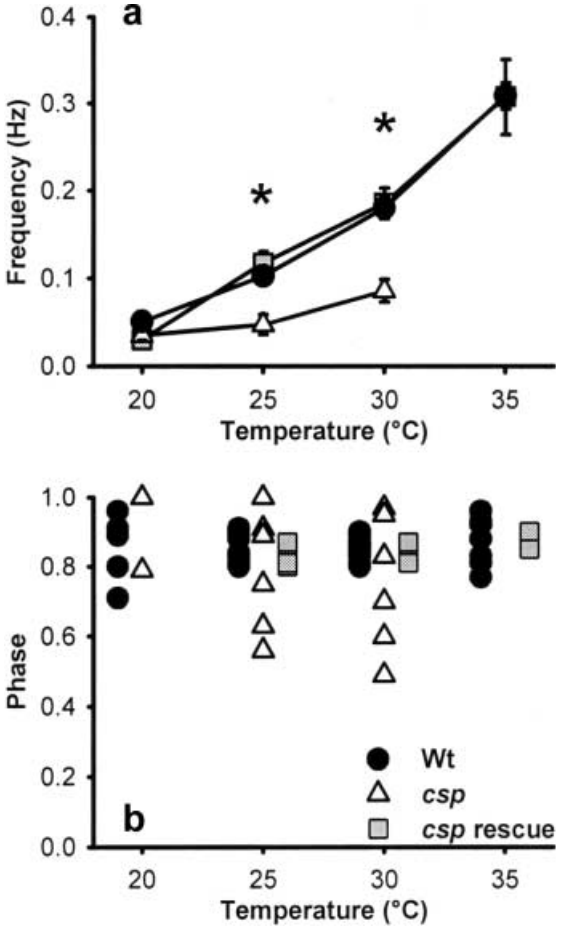

Fig. 7a,b Slower, uncoordinated central locomotor rhythms in *Drosophila csp* null mutants. **a** Motor rhythm frequency was decreased in *csp* null mutants with respect to wildtypes. Mutant larvae had greatly reduced temperature sensitivity of burst frequency, whereas reinsertion of the *csp* gene (*csp* rescue) restored wildtype temperature sensitivity. Significant differences in burst frequency occur between Wt and *csp* at 25°C (*t*-test, $t = 4.26$, $df = 25$, $P < 0.01$)) and 30°C ($t = 4.76$, $df = 29$, $P < 0.01$) and between *csp* rescue and *csp* at 25°C ($t = 4.24$, $df = 12$, $P < 0.01$) and at 30°C ($t = 4.73$, $df = 12$, $P < 0.01$). No significant difference occurred between Wt and *csp* rescues ($P > 0.05$). Note that error bars are mostly hidden by the graph symbols. **b** Intersegmental phase delays between CNS-generated bursts of activity in segments 3 and 5 of muscle 6 were tightly coordinated in wildtype and *csp* rescue preparations. In *csp* mutants, the variability in pattern phasing was increased ($P < 0.01$); however, phasing was temperature insensitive in all flylines (Wt, *csp*, and *csp* rescues; $P > 0.05$). Delay was determined from the peaks of the burst in segment 5 and segment 3, and expressed as a fraction of total cycle duration. Mean phase of the motor rhythm in segment 3 within the period of the motor rhythm in segment 5 was 0.85 in all flylines. There were no significant differences between flylines, assessed with Mann-Whitney rank sum tests (Wt versus *csp*; *csp* versus *csp* rescue; $P > 0.05$) or *t*-test (Wt versus *csp* rescue; $P > 0.05$)

Discussion

Speculation on possible mechanistic roles for CSP during regulated exocytosis at the neuromuscular junction has generated much controversy (Umbach et al. 1998; Dawson-Scully et al. 2000). The present results extend the physiological characterization of CSP to synaptic transmission within the CNS and identify a critical role for it in endogenous motor pattern generation. Reinsertion of the *csp* gene completely rescued the mutant phenotype, indicating that the quantifiable defects in CNS activity can be attributed to the null mutation of *csp*. These results, together with the demonstration that the deletion of *csp* exerts a negative effect on the activity of the CNS at all temperatures, are consistent with a functional role for CSP in central synaptic transmission similar to that seen at the neuromuscular junction.

The motor rhythms described here are comparable to those of an independent description in *Drosophila* larvae using single electrode recording of ventral abdominal muscle 6 (Cattaert and Birman 2001). Our experimental protocol, however, recorded simultaneously from homologous muscles in different segments on opposite sides of the body midline. The demonstration of synchronicity of bursting activity between the left and right sides of the body with the appropriate phasing indicates that the rhythmic motor activity is representative of a coherent locomotor pattern generated by the larval CNS (Marder and Calabrese 1996; Skinner and Mulloney 1998) and likely underlies peristaltic movement of the *Drosophila* larvae. Using the *Drosophila* larval preparation for the investigation of central pattern generation provides an opportunity for the molecular genetic dissection of neural function in the CNS. With the recent completion of the *Drosophila* genome sequence many genes have been discovered which code for integral components of synaptic transmission and for other proteins thought to influence synaptic transmission. The type of experimental approach described here can be used to monitor the operation of the CNS in *Drosophila* models of human disease and neural dysfunction.

The temperature range examined in these experiments is consistent with the thermal environment naturally encountered by the *Drosophila* larvae (Feder et al. 1996). The effect of temperature to increase locomotor rhythm frequency has been well described in other organisms (Montgomery and Macdonald 1990; Janssen 1992); thus, a similar result in *Drosophila* is not surprising and the marked reduction in the slope of the rhythm frequency/temperature relationship in *csp* mutants at permissive temperatures indicates impaired locomotor circuit function. Although the oscillatory circuit responsible for larval locomotion is still unknown, temperature is known to have a profound influence on

intrinsic network dynamics which contribute to setting rhythm frequencies of the CNS in other invertebrate and vertebrate preparations (Marder and Calabrese 1996; Calabrese 1998). Considering that consistent, tight intersegmental phasing is essential for the production of coordinated rhythmical movement (Skinner and Mulloney 1998), it is perhaps critical for this poikilotherm that phase delay is resistant to ecologically-relevant increases in temperature. However, mutant preparations had a lower success rate for CNS rhythms, which became increasingly irregular at elevated temperatures. Only those mutant preparations with successful rhythms and well-defined phasing can be included in the statistical analysis. Thus, an effect of the mutation on intersegmental phasing would be underestimated and the lack of statistical significance in the difference between *csp* and wildtype is misleading. These results demonstrate that whereas rhythmical bursts of activity are possible in *csp* null mutants, the generation, timing and precise coordination of the output of the larval CNS is negatively affected, both at normal and elevated temperatures.

The precise physiological role for CSP at the synapse is not fully understood; however, maintenance of CNS bursting activity at nonpermissive temperatures reinforces the concept that Ca^{2+} entry during regulated exocytosis is not completely attenuated. The mechanisms involved in rhythm generation in *Drosophila* remain unknown. An effect of the mutation on any cellular pacemaker properties of motor patterning within this system certainly cannot be completely ruled out. These results also cannot rule out extrasynaptic effects as a consequence of developmental compensation for the CSP defect, or other indirect effects of the mutation (see for example Renden et al. 2001). Low levels of CSPs have been demonstrated in both neuropil and axons (Eberle et al. 1998); however, the most likely action of CSP is at synapses as it has been primarily characterized as a vesicle-associated protein (Chamberlain and Burgoyne 2000). In addition the temperature-sensitive block of evoked synaptic transmission can be partially rescued by increasing extracellular calcium concentration (Dawson-Scully et al. 2000) which would not be expected to relieve a failure of action potential propagation; the extracellular application of increased concentrations of divalent cations such as calcium decreases axonal excitability (Frankenhaeuser and Hodgkin 1957). Synaptic and pattern-generating network mechanisms, which are known to underlie undulatory locomotion in other vertebrates and invertebrates (Calabrese 1998; Skinner and Mulloney 1998), would be negatively affected by the synaptic defect caused by the mutation. We postulate that it is this defect in synaptic transmission within central rhythm generating circuitry that causes the impairment in motor patterning evident in *csp* null mutants. The frequency of motoneuronal firing during centrally-generated bursts of activity was sufficient to rescue neuromuscular transmission. Thus, somewhat counterinuitively given the original description of the synaptic phenotype of the *csp* null mutant, we conclude that larval paralysis at nonpermissive temperatures is not due to a failure of neuromuscular transmission. Rather we propose that it is due to a failure of locomotor circuit function as a result of inappropriate timing relationships among the elements of the central pattern generator.

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