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Heat Shock–Mediated Thermoprotection of Larval Locomotion Compromised by Ubiquitous Overexpression of Hsp70 in *Drosophila melanogaster*

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Klose, Markus K., David Chu, Chengfeng Xiao, Laurent Seroude, and R. Meldrum Robertson. Heat shock–mediated thermoprotection of larval locomotion compromised by ubiquitous overexpression of Hsp70 in *Drosophila melanogaster*. *J Neurophysiol* 94: 3563–3572, 2005. First published August 10, 2005; doi:10.1152/jn.00723.2005. Maintaining the competence of locomotor circuitry under stressful conditions can benefit organisms by enabling locomotion to more tolerable microhabitats. We show that prior heat shock protects locomotion and the locomotor central pattern generator of larval *Drosophila* against subsequent hyperthermic stress. We combined molecular genetic, electrophysiological, and behavioral techniques to investigate heat shock–mediated thermoprotection. Prior heat shock increased the distance traveled by larvae during hyperthermia before failure. The frequency of the rhythm of peristaltic locomotor contractions and the velocity of locomotion were both less thermosensitive after heat shock and were less susceptible to failure at high temperatures. Rhythmic coordinated motor patterns, recorded intracellularly as excitatory junction potentials in body wall muscles of dissected preparations, were centrally generated because patterns could still be generated in the absence of sensory feedback (sensory function disrupted with *shibire*). Prior heat shock protected central circuit operation during hyperthermic stress by increasing the temperature at which it failed. Overexpression of Hsp70 after a heat shock using transgenic flies (*traII*) did not enhance thermoprotection, as expected, but had deleterious effects on parameters of behavior.

INTRODUCTION

In response to diverse stresses, organisms initiate protective mechanisms enabling survival of subsequent stressors that would otherwise be lethal. For example, during hyperthermic stress the chaperone heat shock protein 70 (Hsp70) is significantly upregulated, suggesting its involvement in thermoprotective mechanisms (for review of Hsps and thermotolerance see Parsell et al. 1993), although many of its actions remain unknown. Preserving the competence of locomotor circuits under stressful conditions can increase an organism's chances of survival by enabling its locomotion to more tolerable microhabitats (Klose and Robertson 2004; Robertson 2004). The inability of arthropods to regulate internal body temperature rapidly and strictly leaves their neurons, and therefore their behaviors, highly susceptible to environmental change (Dawson-Scully and Robertson 1998; Johnson et al. 1991), suggesting that they may provide good models for discovering physiological protective mechanisms.

We used the well-described *Drosophila* larval model, which is amenable to molecular genetic manipulations, and our goal was to examine the hyperthermic stress tolerance of locomotion as well as the consequences of a prior heat shock, with and without Hsp70 overexpression. Hsp70 has been shown to be efficacious in moderating the effects of hyperthermia. For example, respiratory circuit function in mice is particularly susceptible to hyperthermic stress (Tryba and Ramirez 2003, 2004) and synaptic function recorded in the region of the respiratory central pattern generator can be protected against thermal stress by prior thermal conditioning and by exogenous Hsp70 application (Kelty et al. 2002). Also, the upper temperature limit of neuromuscular transmission in *Drosophila* larvae is elevated by prior heat shock (HS) (Karunanithi et al. 1999). The duration of synaptic thermoprotection coincides with the duration of Hsp70 upregulation, although it remains unclear whether Hsp70 has direct protective effects on the operation of neural circuits. Studies have shown that Hsp70 overexpression has no effect on behavior (Le Bourg et al. 2002; Roberts et al. 2003); however, Karunanithi et al. (2002) showed that Hsp70 overexpression does confer enhanced protection of neuromuscular transmission.

Molecular genetic approaches for deciphering mechanisms of central pattern generation are receiving considerable attention (Kiehn and Kullander 2004; Kullander 2005), and such approaches have been notably successful for *Drosophila* larval locomotor patterning (Suster and Bate 2002; Suster et al. 2003). Ours is the first study to combine molecular genetic, electrophysiological, and behavioral techniques to investigate HS-mediated thermoprotection of neural circuits and behavior. Locomotor function was assessed by measuring the distance traveled under hyperthermic conditions and by examining the intersegmental kinematics during a temperature ramp. Intracellular recordings taken from abdominal muscle 6 monitored fictive locomotor output in a dissected preparation during a temperature ramp. Engineered larvae with 12 extra copies of the Hsp70 gene (Welte et al. 1993) were used to examine the effects of HS-induced Hsp70 overexpression.

We describe rhythmic coordinated motor output from muscles and provide further evidence that these recordings were output from a locomotor central pattern generator. We also found that prior HS protected locomotion and central circuit operation during hyperthermic stress, but that ubiquitous overexpression of Hsp70 after HS did not enhance such thermoprotection, but instead we saw an unexpected deleterious effect on behavior.

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METHODS

Fly stocks

Canton-S larvae were raised on a molasses-based fly food at 25°C and used to test for HS-induced thermoprotection of locomotion and locomotor circuit output.

To investigate the role of Hsp70 in thermotolerance we used two engineered fly lines, one containing 12 extra copies of Hsp70 (*traII*) and the control (*cisII*) with only the flanking P-element at the same site (Welte et al. 1993).

The *traII* and *cisII* lines of *Drosophila melanogaster* are extra copy and excision strains. Transgene insertion is into chromosome II. The extra copy strain *traII* contains 12 extra copies of the Hsp70 gene, an eye-color marker (*whs*), and flanking yeast recombination targets and P-elements. The excision strain *cisII* shares the same chromosomal sites of transgene insertion and flanking sequences but lacks the extra copies of the Hsp70 gene and eye-color marker.

Upstream activating sequence (UAS) temperature-sensitive *shibire* (shi^{ts1}) on the third chromosome (Kitamoto 2001) were used for the sensory knockout experiments and were crossed with the PO163-Gal4 driver line, which targets the peripheral nervous system (Hummel et al. 2000; Suster and Bate 2002). The *shibire* gene encodes dynamin, a protein involved in vesicle recycling that is necessary for synaptic transmission at moderate temperatures. This experiment examined locomotor output during acute sensory disruption in larvae that developed with normal sensory input.

All experiments used wandering-stage third-instar larvae.

Western blot analysis of heat shock proteins

Nonheat-shocked (NHS) and heat-shocked larvae were collected and stored at −70°C. PAGE was performed in the presence of SDS on 10% gels with a 5% stacking gel using the discontinuous buffer system (Laemmli 1970). The proteins were transferred onto nitrocellulose membrane. For Western analysis of Hsp70, membrane was incubated in primary antibody diluted 1:1,000 (*Drosophila* Hsp70-specific primary antibody; ABR Affinity BioReagents, no. MA3-007), and then secondary antibody (horseradish peroxidase–conjugated IgG; Stressgen no. SAB-100) diluted 1:4,000. Immunoreactive bands were visualized by use of ECL Advance Western Blot Detection Kit (RPN2135, Amersham), and exposed to Kodak BioMaxFilm. Quantification was done using the ChemiGenius BioImaging System (Syngene), with Gene Tools software.

Heat shock

Larvae were placed in petri dishes containing moistened tissue and sealed to preserve humidity. The sealed dish was placed in an oven at 36°C for 1 h. After HS, larvae were allowed to recover at room temperature for approximately 60 min before experiments were undertaken. Western blots confirmed the heat shock–induced Hsp70 overexpression phenotype described in *traII* lines (Fig. 1, *A* and *B*).

Kinematic analysis

Larvae were placed in a glass dish (5.5 cm diameter) containing 10 ml of hardened 3.2% nonnutritive agar solution. NHS control and HS larvae were videotaped during periods of linear forward crawling as the temperature of the agar was ramped from room temperature at a rate of about 1°C/min. The temperature was controlled using a peltier plate (SH 1.4-125-045L, Melcor) placed under the dish. The temperature of the agar was measured by inserting a thermocouple (0.2 mm; BAT-12, Sensortek) just below the surface. Locomotion was videotaped using a CCD camera mounted on a stereomicroscope (PZMTIII, World Precision Instruments) and recordings were made at a sample rate of 30 frames/s. The digitized videos were analyzed using Videopoint motion analysis software (LSW Software). Displacement patterns of the head, tail, and third and fifth abdominal segments were measured.

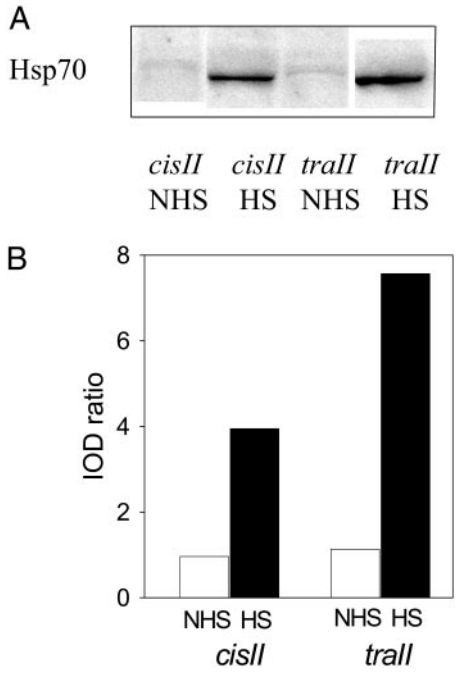

FIG. 1. Expression of chaperone heat shock protein 70 (Hsp70) in *Drosophila* larvae after heat shock. Whole wandering third-instar larvae were heat shocked (1 h at 36°C) then allowed to recover at room temperature (21–24°C) for 1 h. *A*: Western immunoblots of whole body larval lysates using 10 larvae in each test group detected Hsp70. *B*: quantification of Hsp70 using intraoptical densities (IODs) confirmed heat shock–induced overexpression of Hsp70 in *traII* compared with *cisII* (see text for details of procedures).

The frequency of peristaltic locomotor contractions was calculated by measuring the time period between the peaks of displacement of the head segment while the larvae crawled. Using the methodology of Berrigan and Pepin (1994), the crawling velocity was determined by taking a linear regression of the displacement versus time data for the tail segment. The phasing of abdominal segments was determined by measuring the time delay between activity in the third and fifth segments and then expressing this time delay as a fraction of the total cycle duration.

Wandering distance analysis

Larval locomotor ability was further assessed by measuring the distance traversed by the larvae during three consecutive 5-min time intervals. A clear acetate sheet was placed over top of the agar dish and the path of the larvae was traced on the sheet at both 20 and 42°C. The tracings were then digitized and the path lengths were measured using the ImageJ software package (National Institutes of Health, rsb.info.nih.gov/ij/).

Electrophysiology

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 a modified hemolymph-like solution (HL3) with 70 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 115 mM sucrose, 5 mM trehalose, at pH 7.6 (Stewart et al. 1994). Dual intracellular recordings were made with glass microelectrodes filled with 1 M KCl. Intracellular recordings were amplified using an Axoclamp-2A amplifier (Axon Instruments, Foster City, CA) ein bridge mode and recorded with the Digidata 1322A-Axoscope

acquisition system. To assess the thermosensitivity of CNS output, recordings were made from abdominal muscle 6 of segments 3 and 5. A dual-electrode arrangement permits a precise characterization of the locomotor pattern phasing between and within segments. Intersegmental phasing was assessed using the time delay between the peak burst frequency of segments 3 and 5. Intrasegmental phasing was assessed relative to motor bursts in muscle 6. Four consecutive bursts in at least three animals were used for each muscle. Any activity <0.5 s in duration was not used in the assessment of segmental phasing. To test the thermosensitivity of motor pattern generation, recordings of locomotor patterns (Barclay et al. 2002; Cattaert and Birman 2001) were made while the temperature of the saline was ramped at 1°C/min until coordinated rhythmic motor bursting ceased.

Iontophoresis

Glutamate was iontophoretically applied to the exposed larval body-wall muscle number 6 as described by Klose et al. (2002) while intracellular recordings of potential changes were taken. The iontophoresis electrode contained 1 M L-glutamic acid.

Sensory knockout analysis

To assess the propensity for locomotor activity (i.e., the "willingness" of the larvae to locomote measured as the % of total recording time exhibiting motor activity) the total duration of motor activity was measured in each of three temperature bins, room temperature to 29, 29–33, and 33–40°C, and then divided by the total duration of each bin. Below 29°C *shibire* mutants are known to be functionally normal. At 29°C vesicle recycling is disrupted, although a time lag occurs between cessation of vesicle recycling and functional disruption of transmission arising from an existing pool of vesicles. At 33°C during a 1°C/min temperature ramp motor activity decreased substantially, indicating *shibire* silencing of sensory neuron activity.

Statistical analysis

Data sets for whole animal locomotor assays used nine or ten individuals in each treatment group. For other comparisons Ns are noted in the text. Most statistical tests were performed using SigmaStat (v3.0) (SPSS, Chicago, IL). Data were tested for normality and equal variance and two-way repeated-measures (RM) ANOVAs were used to test for significance unless otherwise stated. Any pairwise multiple comparisons were subsequently performed using the Holm–Sidak method. Significance was assessed at $P < 0.05$ and data are presented as means ± SE.

For the analysis of data shown in Fig. 5, we used JMP IN (v5.1) to perform RM ANOVA with three between-subject factors (temperature, genetic strain, and HS treatment) and one within-subject factor (time) using the REML (restricted maximum likelihood) method so that the SEs would be properly scaled. To determine where the main effects occurred, separate two-way RM ANOVAs were then run on the data for each of the graphs in Fig. 5, *A–D*. Similar procedures were used to test the data of Fig. 6.

RESULTS

Thermosensitivity of larval locomotor behavior

Locomotor competence of NHS and HS Canton-S larvae during hyperthermia was assessed by measuring the distance traveled over three consecutive 5-min periods (Fig. 2*A*). A three-way ANOVA (between-subject factors: HS pretreatment, temperature, time bin) on all the data illustrated in Fig. 2, *B* and *C* showed significant main effects of HS ($F = 8.0$, $P = 0.006$, degrees of freedom [df] = 1, 96), temperature ($F = 56.4$, $P < 0.001$, df = 1, 96), and time ($F = 24.6$, $P < 0.001$, df = 2, 96). There were also significant interactions between HS and temperature ($F = 11.8$, $P < 0.001$, df = 1, 96) and between temperature and time ($F = 35.3$, $P < 0.001$, df = 2, 96), indicating that the effects of HS and time depended on the temperature of the assay (compare Fig. 2*B* with Fig. 2*C*). We subsequently analyzed the data of Fig. 2, *B* and *C* separately using two-way RM ANOVAs. At room temperature there were no

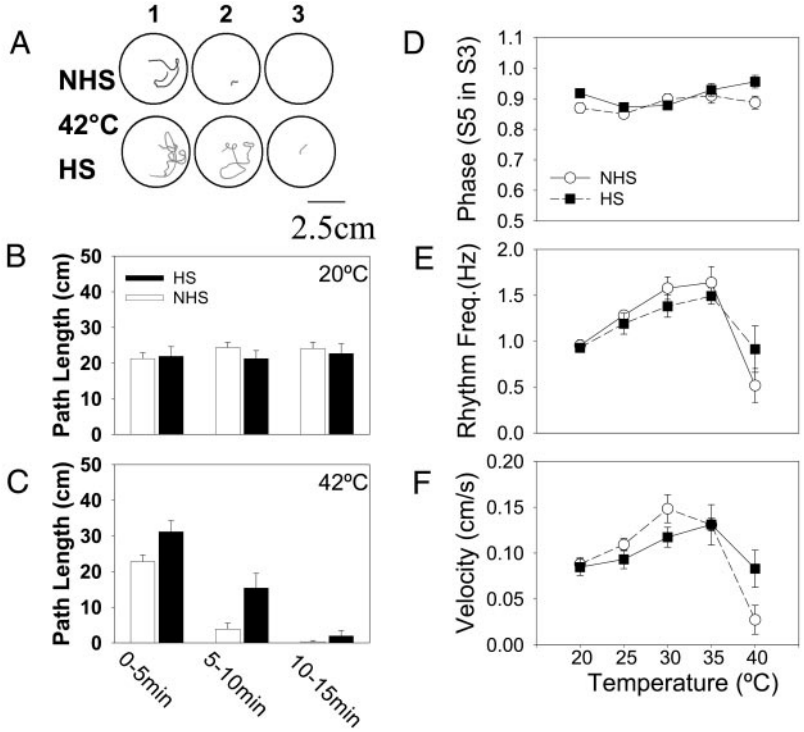

FIG. 2. Larval behavior is protected by a prior heat shock. *A*: sample wandering paths from 3 consecutive 5-min periods (labeled 1, 2, and 3), in one nonheat-shocked (NHS) and one heat-shocked (HS) larva, at 42°C. Note longer path lengths for the HS larva particularly in the 2nd 5-min period (2). *B*: at room temperature (RT) there was no difference in distance traveled between HS and NHS larvae. *C*: at 42°C HS larvae traveled farther than NHS larvae in each time bin. *D*: phase relationship between movements of segments 3 (S3) and 5 (S5) during locomotion was not temperature sensitive. *E*: peristaltic rhythm frequency increased with increasing temperature ≤35°C above which it decreased. NHS larvae were more temperature sensitive than HS larvae. *F*: locomotor velocity increased with increasing temperature ≤30–35°C above which it decreased. NHS larvae were more temperature sensitive than HS larvae by peaking at a lower temperature and having a greater reduction at the highest temperature. In this and all subsequent figures graph symbols represent means ± SE. Statistical details in text.

significant differences between NHS and HS path lengths during any of the three time periods (Fig. 2*B*). The total distance traveled by larvae over the first 5 min at room temperature was 21.3 ± 1.7 cm for NHS larvae and 21.9 ± 2.7 cm for HS larvae. At 42°C, however, HS larvae significantly outperformed NHS ($F = 23.5$, $P < 0.001$, df = 1, 16) and path length decreased with increasing time in the assay ($F = 56.2$, $P < 0.001$, df = 2, 32) (Fig. 2*C*). There was no interaction between HS and time, indicating that HS affected distance traveled similarly in each of the three time bins. During the first 5 min NHS larvae traveled 22.8 ± 1.9 cm, whereas HS larvae traveled significantly farther, averaging 31.2 ± 3.1 cm. The largest effect of HS was in the second 5 min when HS larvae traveled almost four times farther than NHS larvae (15.4 ± 4.1 cm vs. 3.9 ± 1.6 cm). In the last 5 min NHS larvae traveled 0.3 ± 0.3 cm, whereas HS larvae traveled 2.0 ± 1.5 cm.

Various parameters of locomotor kinematics were measured during an increasing temperature ramp to assess thermosensitivity and to test for the protective effects of a prior HS. Intersegmental phasing was not affected by temperature or by a prior HS because bursts in segment 5 remained phase-locked at approximately 0.9 relative to cycles recorded in segment 3 across the temperature range (Fig. 2*D*).

The frequency of peristaltic contractions during locomotion increased with increasing temperature and peaked at about 1.5 Hz around 35°C before precipitously declining at 40°C (Fig. 2*E*; main effect of temperature: $F = 23.7$, $P < 0.001$, df = 4, 72). There was no main effect of HS but there was a significant interaction between HS and temperature ($F = 2.6$, $P = 0.043$, df = 4, 72), indicating that HS affected the thermosensitivity of locomotor rhythm frequency. HS larvae did not have as extreme a reaction to the temperature ramp as NHS larvae. In NHS larvae the peak frequency was higher at 1.64 Hz and dropped by more than 1 Hz to 0.52 Hz at 40°C, whereas in HS larvae the peak frequency was 1.49 Hz and it dropped by only 0.58 to 0.91 Hz at 40°C (Fig. 2*E*).

The velocity of locomotion also increased with increasing temperature, peaking around 0.14 cm/s (mean of NHS and HS values) before declining at higher temperatures (Fig. 2*F*; main effect of temperature: $F = 17.6$, $P < 0.001$, df = 4,72). There was no main effect of HS but there was a significant interaction between HS and temperature ($F = 4.5$, $P = 0.003$, df = 4,72), indicating that HS affected the thermosensitivity of the velocity of larval locomotion. In a similar fashion as for frequency, the locomotor velocity of HS larvae was less sensitive to the temperature ramp. In NHS larvae the peak speed was 0.15 cm/s at 30°C, whereas HS larvae had a lower peak of 0.13 cm/s at a higher temperature of 35°C. Moreover, in NHS larvae the velocity dropped from its peak by 0.12 to 0.03 cm/s at 40°C, whereas in HS larvae the drop from the peak was only 0.05 to 0.08 cm/s at 40°C (Fig. 2*F*).

In summary, HS had no significant effect on rhythm frequency, velocity, or distance traveled at permissive (nonstressful) temperatures (20–25°C); the effect of HS was evident only at higher, more stressful temperatures (>30°C). HS induced thermotolerance of locomotion by attenuating the detrimental effects of hyperthermic stress on velocity, rhythm frequency, and distance traveled.

Characterization and thermosensitivity of motor pattern generation

To examine CNS locomotor output more directly, larvae were dissected and intracellular recordings were taken from body-wall muscles. Rhythmically coordinated motor output recorded intracellularly from body-wall muscles in the form of excitatory junction potentials was used to characterize fictive locomotion in the fillet preparation as well as to monitor central pattern generation during a hyperthermic stress. Fictive locomotor recordings show muscle 6 of segment 5 phase-locked at about 0.9 relative to cycles recorded in muscle 6 of segment 3 (Fig. 3*A*), which fits with previous publications of forward fictive locomotion (Barclay et al. 2002). This phase relationship is temperature insensitive. The phase relationship of intrasegmental coordination was also temperature insensitive. Figure 3*B* shows simultaneous motor activity recorded from m6 and m23, both in segment 3. An intrasegmental motor pattern was characterized by dual intracellular recordings such that the activities of different segmental muscles were measured relative to the activity of muscle 6. No single preparation yielded recordings from all muscles but in some instances it was possible to monitor more than one muscle sequentially and compare their activities with that of muscle 6. A pattern for 13 segmental muscles was reconstructed (Fig. 3*C*).

The rhythm frequency of fictive locomotion was much slower than the rhythm frequency observed in freely moving intact larvae. In an effort to account for this reduction, whole larvae were restrained and allowed to attempt locomotion. Rhythm frequencies were compared for free larvae ($n = 9$), restrained larvae ($n = 6$), and the fillet preparation ($n = 9$) at different temperatures (Fig. 3*D*). Rhythm frequency was significantly affected by the type of preparation ($F = 129.1$, $P < 0.001$, df = 2, 21) and by the temperature ($F = 19.3$, $P < 0.001$, df = 1, 18) but there was no interaction between these factors. The rhythm frequency of restrained whole larvae was significantly reduced from 0.95 ± 0.06 Hz while freely moving to 0.12 ± 0.02 Hz by pinning ($t = 13.0$, $P < 0.001$, df = 21) and to 0.11 ± 0.01 Hz by filleting ($t = 14.2$, $P < 0.001$, df = 21). There was no significant difference between the effects on rhythm frequency of pinning and filleting, which suggests that the reduction of rhythm frequency was the result of the physical restraint, and was not a deleterious result of dissection.

To confirm that the locomotor patterns were centrally generated and to rule out disrupted sensory feedback as a cause of pattern failure, temperature-sensitive *shibire* was driven in sensory neurons using the PO163-Gal4 driver, which prevented sensory feedback at moderate temperatures. Larvae lacking sensory input revealed motor patterns with normal phasing (Fig. 3*E*), yet the propensity for motor activity was strikingly reduced (Fig. 3*F*). In *shibire* mutants, dynamin, a vesicle recycling protein, is sensitive to temperatures >29°C, which ultimately leads to inhibition of transmitter release in 3 to 4 min (Kitamoto 2001; Ramaswami et al. 1993). Motor patterns were recorded in three temperature ranges (room temperature [RT]–29, 29–33, 33–40°C) for two control strains (driver alone, $n = 3$; UAS alone, $n = 5$) and the experimental strain (*shibire* driven in sensory neurons, $n = 6$). There was an effect of temperature on the propensity to generate locomotor patterns ($F = 17.7$, $P < 0.001$, df = 2, 17) but no main effect of genetic strain. However, there was also a significant inter-

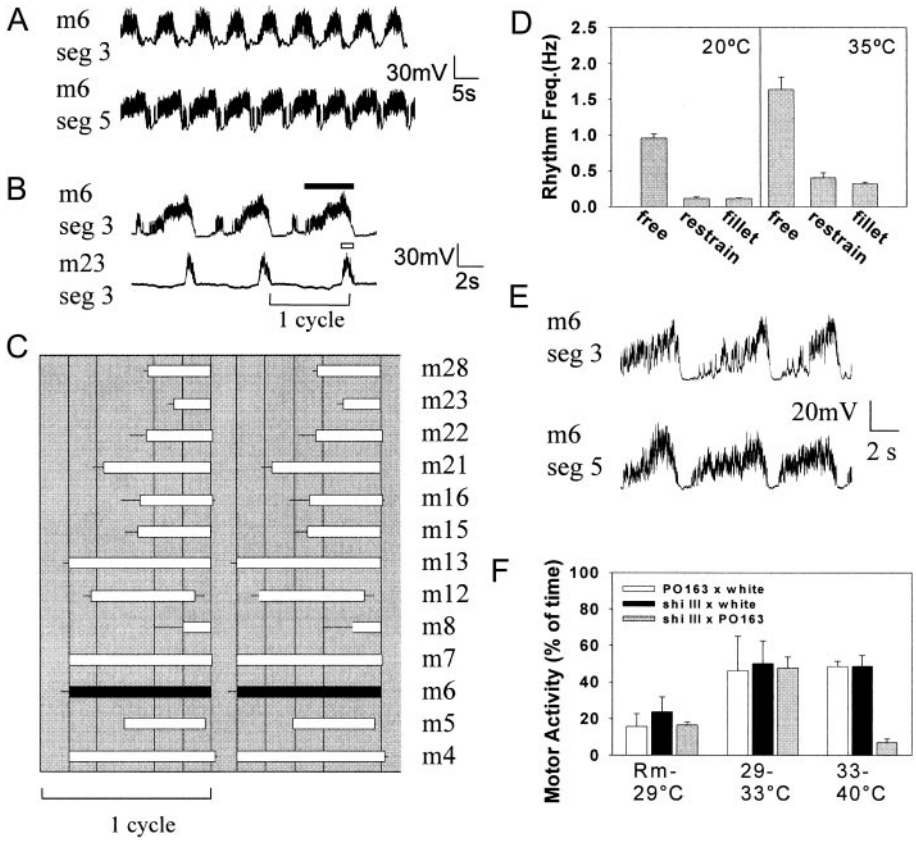

FIG. 3. Rhythmically coordinated motor output recorded in muscles is centrally driven. *A*: intracellular recordings taken from muscle 6 in segment 3 (*top*) and segment 5 (*bottom*) at 29.5–31°C show coordination of activity between segments during the expression of a locomotor pattern. *B*: intracellular recordings taken from muscle 6 (*top*) and muscle 23 (*bottom*) in segment 3 show coordination of activity within a segment. *C*: schematic representation of intrasegmental coordination of motor activity of various accessible muscles. Boxes represent the average burst duration of each muscle recorded relative to the burst recorded simultaneously in muscle 6 shown in black. Bars represent the SD. Two identical cycles are shown to illustrate the rhythm. *D*: locomotor rhythm frequency increased with increasing temperature in freely moving larvae, restrained whole larvae, and dissected fillet preparation. Note that restrained whole larvae and dissected fillet preparations had significantly slower rhythms than those of freely moving larvae. *E*: motor pattern recording from muscle 6 in segments 3 and 5 showing typical fictive locomotor bursts after sensory knockout at 34.5°C using *shibire*. Motor patterns failed at 37.5°C in this preparation. *F*: propensity for preparations to generate locomotor activity (percentage of the total recorded time in which motor activity occurs) increased with increasing temperature. In the first 2 temperature bins (Rm.–29 ° and 29–33°C) no difference in propensity was seen between larvae containing upstream activating sequence (UAS) *shi*^{ts1} on the third chromosome and the P0163-Gal4 driver [progeny from cross between w^{1118} (PO163) and w^{1118} (UAS *shi*^{ts1})], and the two control lines, UAS alone (progeny from cross between $w^{1118} \times w^{1118}$; UAS-*shi*^{ts1}) and Gal4 alone (progeny from $w^{1118} \times w^{1118}$; PO163). Propensity for motor activity decreased significantly at temperatures above 33°C in *shi*^{ts1} when crossed with P0163. Experiments using UAS *shi*^{ts1} on the second chromosome driven by P0163-Gal4 showed the same significant reduction in propensity for motor output (data not shown). Statistical details in text.

action between temperature and strain ($F = 4.4$, $P = 0.013$, df = 4, 17), indicating that the effect of genetic strain was thermosensitive (Fig. 3*F*). Before the manifestation of *shibire*'s effects, rhythm frequency and phasing were not different between lines, nor was the propensity for motor activity. No difference in propensity was seen between the three lines in the first two temperature bins (RT–29 and 29–33°C). However, the propensity for motor activity decreased at temperatures >33°C in larvae containing UAS *shi*^{ts1} driven by PO163-Gal4. Between 29 and 33°C coordinated bursting occurred for 46 ± 5% of the total time. Between 33 and 39°C the propensity for motor activity decreased to 13 ± 3% when *shibire* was expressed in sensory neurons, which was less activity than the UAS control 48 ± 4% and the Gal4 driver control 49 ± 3%. Thus lack of sensory input does not prevent fictive locomotor pattern generation but does reduce its propensity, or its likelihood of occurring. Rhythmic motor patterns can occur in the absence of sensory feedback in most systems, but often at a reduced frequency (Delcomyn 1980). In *Drosophila*, Caldwell et al. (2003) showed that mutant chordotonal sensilla resulted in reduced stride period and persistence of locomotion. Our data reveal that the sensory receptors that are targeted by the P0163 driver were responsible for initiating and maintaining locomotor rhythms but were not responsible for generating the pattern.

As temperature was raised the centrally generated rhythm frequency increased until rhythmic coordinated motor output failed to be elicited (Fig. 4*A*). Prior HS elevated the failure temperature by 2.4°C from 37.0 ± 0.4°C in NHS ($n = 7$) to 39.4 ± 0.5°C in HS larvae ($n = 6$) ($t = 3.89$, $P = 0.003$, df = 11) (Fig. 4*B*).

Intraburst peak frequency of excitatory junctional potentials was measured in NHS larvae over the peak 0.02 s of each burst within temperature bins. Before failure, intraburst peak frequency in NHS larvae ($n = 6$) decreased from 114.3 ± 7.0 to 87.9 ± 4.2 Hz and was 27.1 Hz lower than that in HS larvae ($n = 4$) at the same temperature (125.0 ± 2.0) ($t = 6.7$, $P < 0.001$, df = 8) (Fig. 4*C*). That peak burst frequency began to

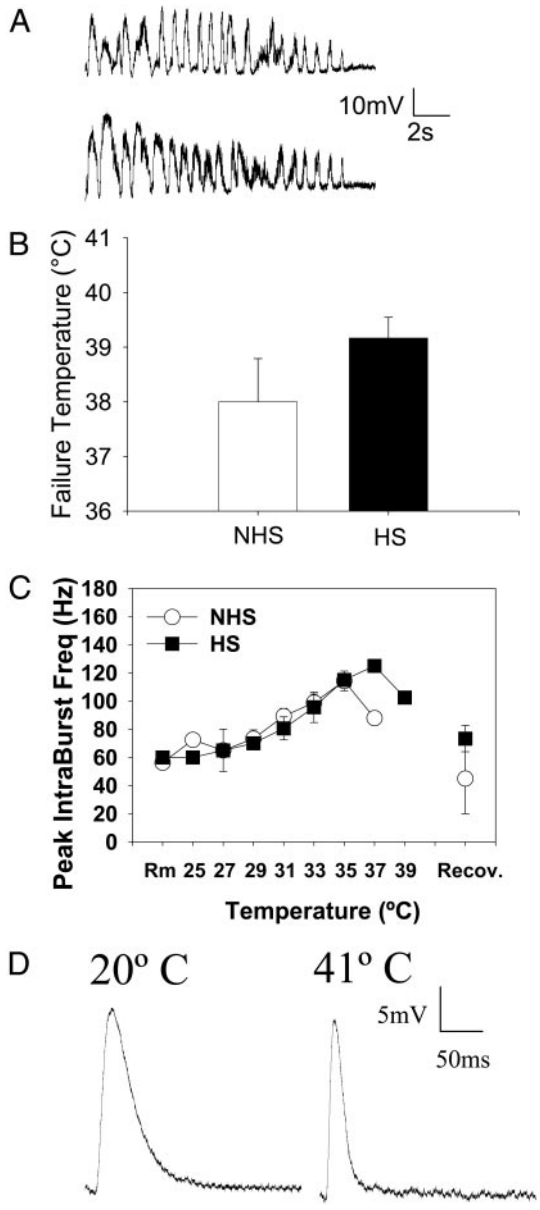

FIG. 4. Thermotolerance of motor pattern generation is enhanced by prior heat shock. *A*: simultaneous recordings of muscle activity show failure of locomotor circuit output. Temperature was increased at 1°C/min until rhythmic coordinated motor output failed to be elicited (37.5°C in this preparation). *B*: prior HS significantly increased the temperature at which locomotor rhythms failed compared with NHS larvae. *C*: peak intraburst frequency assessed over a 0.2-s duration increased with increasing temperature until 35°C and then decreased before failing. HS larvae better maintained peak intraburst frequency at 37°C. After returning to RT peak intraburst frequency in both HS and NHS return to pretemperature ramp levels (Recov.). *D*: representative traces of L-glutamate–induced potentials recorded from segmental muscle 6 in NHS larvae at RT and at 41°C. Note that in the fillet preparation locomotor circuitry fails before reaching 41°C (see *B*). Statistical details in text.

decline before failure occurred indicates that mechanisms before muscle activation were being disrupted.

We examined the thermosensitivity of glutamate receptor function by measuring the thermosensitivity of L-glutamate–induced potentials. We found that glutamate potentials can still be elicited at temperatures higher than any rhythmically coordinated motor patterns (Fig. 4*D*), indicating that glutamate receptor function at the neuromuscular junction was still operational after the hyperthermic failure of motor patterns.

Overexpression of Hsp70 does not confer enhanced protection against hyperthermia

Much interest has been given to the role of Hsp70 involvement in stress protection, including protection from hyperthermic stress. Ubiquitous overexpression of Hsp70 in *Drosophila* has significant effects on neuromuscular transmission that are thought to be protective (Karunanithi et al. 2002). We used the same two *Drosophila* lines as in this previous study: *traII*, which has 12 extra copies of Hsp70, and *cisII*, the insertional control, and we expected that the *traII* flies would outperform the *cisII* flies at high temperatures after heat-shock pretreatments. We examined the thermosensitivity of the same parameters of locomotor behavior as above for NHS and HS Canton-S flies. Statistical analysis of all the data presented in Fig. 5, *A–D* revealed significant interactions between:

1) HS and the temperature of the assay ($F = 9.9$, $P = 0.002$, df = 1, 74)

2) genetic strain and HS ($F = 6.0$, $P < 0.02$, df = 1,74)

3) temperature and time period ($F = 148.3$, $P < 0.0001$, df = 2, 148)

4) genetic strain and time period ($F = 3.3$, $P = 0.04$, df = 2, 148)

5) time period and temperature and HS ($F = 6.8$, $P = 0.001$, df = 2, 148)

These interactions are visible on the graphs but, because of the significant interaction terms, the main effects are difficult to interpret as a whole. Thus we ran separate two-way RM ANOVAs on each of the graphs of Fig. 5.

For NHS larvae at room temperature (Fig. 5*A*) there was no significant effect of genetic strain ($F = 0.57$, $P = 0.46$, df = 1, 18), or of time ($F = 3.1$, $P = 0.06$, df = 2, 36), and no interaction between these two factors ($F = 0.57$, $P = 0.58$, df = 2, 36). At room temperature after HS, however, there was a significant effect of genetic strain ($F = 4.7$, $P = 0.04$, df = 1, 20) but no effect of time ($F = 0.9$, $P = 0.4$, df = 2, 40) and no significant interaction ($F = 0.06$, $P = 0.95$, df = 2, 40). It can be seen that HS *cisII* larvae traveled farther than HS *traII* larvae at room temperature, although there was no variation in the path length over time (Fig. 5*B*).

When the locomotor assays were performed at 42°C with NHS larvae (Fig. 5*C*), there was a significant effect of genetic strain ($F = 9.7$, $P = 0.006$, df = 1, 18) and of time ($F = 235.4$, $P < 0.0001$, df = 2, 36) and a significant interaction between these factors ($F = 9.0$, $P = 0.0007$, df = 2, 36). Separate RM ANOVAs for each strain showed highly significant variation with time ($P < 0.0001$ for each; other statistical parameters not shown). Thus the thermosensitivities of NHS *cisII* and *traII* larvae were different, and the path lengths of *cisII* larvae were longer than those of *traII* larvae in each time period (Fig. 5*C*). After the HS the results were similar, although both strains traveled farther in each time period (Fig. 5*D*). There was a significant effect of genetic strain ($F = 15.2$, $P = 0.001$, df = 1, 18) and of time ($F = 203.1$, $P < 0.0001$, df = 2, 36), and a significant interaction between these factors ($F = 3.8$, $P = 0.03$, df = 2, 36). Separate RM ANOVAs for each strain showed highly significant variation with time ($P < 0.0001$ for each; other statistical parameters not shown). Thus contrary to

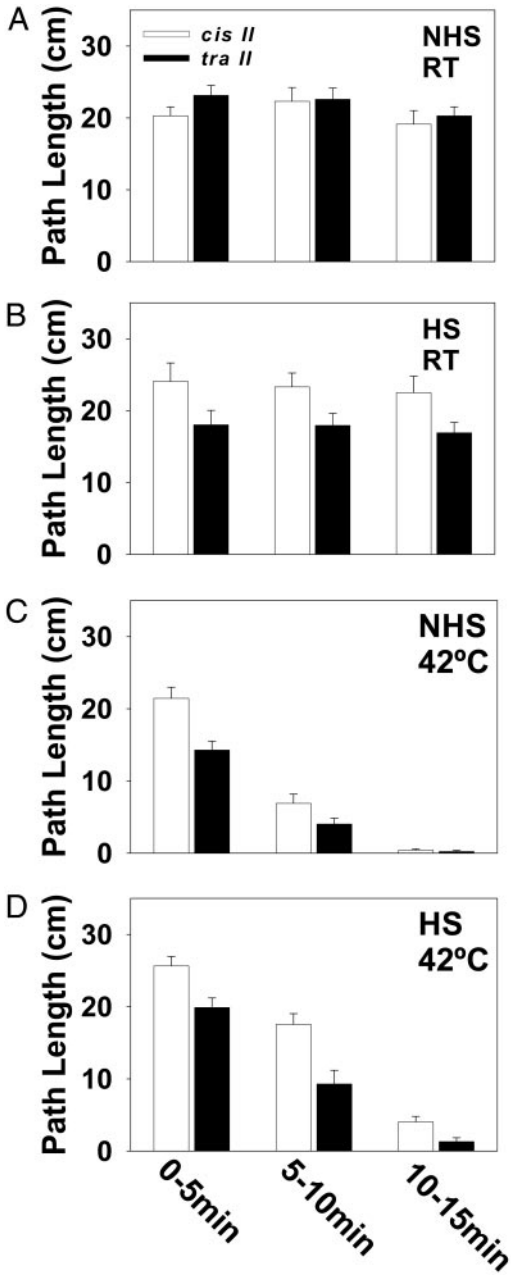

FIG. 5. Overexpression of Hsp70 does not enhance protection by increasing distance traveled at high temperatures. *A*: distance traveled at RT was not different between NHS *cisII* and *traII* larvae. *B*: *cisII* larvae traveled farther than *traII* larvae at RT 60 min after recovery from HS. *C*: at 42°C NHS *cisII* traveled farther than NHS *traII* larvae and for both strains the distance traveled decreased with increasing time. *D*: at 42°C HS *cisII* traveled farther than HS *traII* larvae and for both strains the distance traveled decreased with increasing time. Statistical details in text.

our expectations, at 42°C after the HS, *cisII* larvae traveled farther than *traII*.

A statistical analysis of the effect of HS on the thermosensitivity of central locomotor rhythm frequencies of *cisII* and *traII* larvae (Fig. 6, *A* and *B*) revealed significant effects of:

1) genetic strain ($F = 5.7$, $P = 0.02$, df = 1, 36)

2) HS ($F = 5.5$, P = 0.02, df = 1, 36)

3) temperature ($F = 19.4$, $P < 0.0001$, df = 4, 141)

4) the interaction between temperature and HS ($F = 8.8$, $P < 0.0001$, df = 4, 141)

A separate two-way RM ANOVA on the data from NHS larvae (Fig. 6*A*) showed an effect of temperature ($F = 17.6$, $P < 0.0001$, df = 4, 72) but no significant effect of strain ($F = 2.2$, $P = 0.16$, df = 1, 18) and no interaction between temperature and strain ($F = 2.5$, $P = 0.051$, df = 4, 72). This was also true for the data from HS larvae (Fig. 6*B*) that showed an effect of temperature ($F = 8.5$, $P < 0.0001$, df = 4, 72) but no significant effect of strain ($F = 3.5$, $P = 0.08$, df = 1, 18) and no interaction between temperature and strain ($F = 0.47$, $P = 0.75$, df = 4, 72). Thus the effect of HS on the thermosensitivity of rhythm frequency, described above for Canton-S

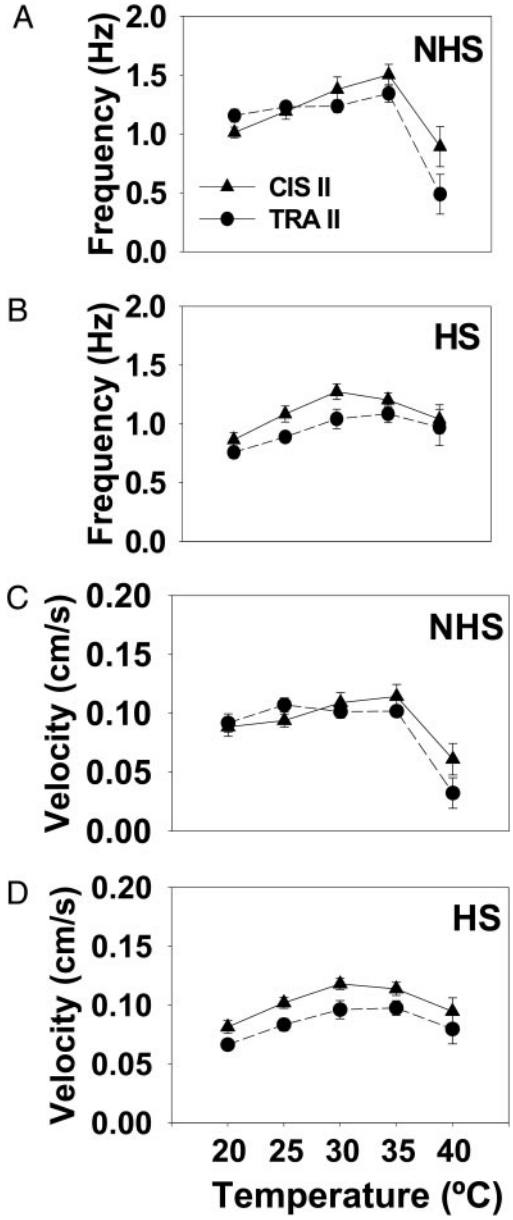

FIG. 6. Overexpression of Hsp70 does not enhance protection of rhythm frequency or locomotor velocity at high temperatures. *A*: in both NHS *cisII* and NHS *traII* larvae the frequency of locomotor contractions increased as temperature increased up to 35°C. *B*: after HS the rhythm frequencies of *cisII* and *traII* larvae were less thermosensitive (compare with *A*) and *traII* larvae tended to have lower frequencies. *C*: in both NHS *cisII* and NHS *traII* locomotor velocity increased as temperature increased up to 35°C. *D*: after HS the velocities of *cisII* and *traII* larvae were less thermosensitive (compare with *C*) and *traII* larvae were slower. Statistical details in text.

larvae, was evident. There was a significant difference between *cisII* and *traII* larvae that should be interpreted with caution because it was evident only in the full analysis. If this is correct, the graphs (Fig. 6, *A* and *B*) indicate that *traII* larvae tended to have lower locomotor rhythm frequencies than those of *cisII* larvae, particularly after HS.

For locomotor velocity a full statistical analysis of the data in Fig. 6, *C* and *D* reveals significant effects of:

1) genetic strain ($F = 5.7$, $P = 0.02$, df = 1, 36)

2) temperature ($F = 23.4$, $P < 0.0001$, df = 4, 140)

3) the interaction between temperature and HS ($F = 9.1$, $P < 0.0001$, df = 4, 140)

There was, however, no main effect of HS ($F = 0.35$, $P = 0.56$, df = 1, 36). A separate two-way RM ANOVA on the locomotor velocity data of NHS *cisII* and *traII* larvae (Fig. 6*C*) showed an effect of temperature ($F = 23.1$, $P < 0.0001$, df = 4, 72) but no effect of genetic strain ($F = 0.65$, $P = 0.43$, df = 4, 72) and no interaction between the two factors ($F = 2.3$, $P = 0.06$, df = 4, 72). After HS (Fig. 6*D*) there was a significant effect of genetic strain ($F = 9.0$, $P = 0.008$, df = 1, 18) and temperature ($F = 8.8$, $P < 0.0001$, df = 4, 72) but no interaction between these factors ($F = 0.10$, $P = 0.98$, df = 4, 72). Thus the effect of HS on the thermosensitivity of locomotor velocity, described above for Canton-S larvae, was evident because in the full analysis the effect of temperature depended on the level of HS. There was also a significant difference between *cisII* and *traII* larvae such that *traII* larvae had lower locomotor velocities than those of *cis* larvae, particularly after HS (Fig. 6, *C* and *D*).

The failure temperature of motor patterns in HS *cisII* ($n = 6$) was 39.8 ± 0.4°C and not different from that of HS *traII* ($n = 5$), 39.3 ± 0.5°C ($t = 0.87$, $P = 0.41$, df = 9). Also, peak intraburst frequency of *cisII* ($n = 6$) at 37°C was 108 ± 11.1 Hz and was not different from that of HS *traII* ($n = 3$) at 86 ± 13 Hz ($t = 1.2$, $P = 0.27$, df = 7).

In summary, overexpression of Hsp70 showed no enhanced thermoprotection of rhythm frequency, velocity, distance traveled, peak intraburst frequency, or failure temperature of motor pattern output. Instead we observed a decrease in performance of some parameters, particularly path length and velocity after HS.

DISCUSSION

Drosophila larval locomotor behavior and fictive locomotor patterns at room temperature in this study are consistent with previous research (Barclay et al. 2002; Berrigan and Pepin 1994; Wang et al. 2002). As temperature increased from room temperature to 35°C, so too did the rhythm frequency and locomotor velocity.

The differences in locomotor rhythm frequencies measured in freely moving larvae and dissected, pinned fillet preparations can be accounted for as an effect of restraint. We provide strong evidence that rhythmic, coordinated motor patterns recorded from body-wall muscles in the larvae indeed represent locomotor output from a central pattern generator as suggested in previous studies (Cattaert and Birman 2001; Suster and Bate 2002).

Prior HS did not alter rhythm frequency, velocity, or distance traveled at nonstressful temperatures, but did allow functional operation at temperatures that otherwise disrupt larval performance. Experiments conducted at 42°C show that distance traveled decreased progressively in NHS and HS larvae across time, but that HS larvae traveled significantly farther than NHS in each time bin. One interpretation might be that a prior HS sensitizes larvae to heat, in an injurious fashion, leading to increased activity. If this were true, it would be expected that, after HS, thermal effects on locomotion would be observed at moderate temperatures (<30°C) as well. Because no effects were seen until the higher temperatures (>30°C), in that for each parameter, HS showed less thermal-induced change compared with NHS and, finally, in that HS rendered locomotor movements less vulnerable to the highest temperature (40°C), it is not likely that HS was deleterious. Thus HS-induced protection of locomotion is manifest as an ability to travel farther and faster at high temperatures and a reduction in the thermosensitivities of rhythmic locomotor contractions and locomotor velocity. Our results demonstrate that a prior HS can reduce the rate at which locomotor ability degrades during hyperthermic stress and that prior HS protects the neural circuitry controlling motor behavior.

By 40°C, during a 1°C/min temperature ramp, dissected NHS larvae lost the ability to generate motor patterns. We have shown that sensory feedback in *Drosophila* larvae was involved in the initiation and maintenance of fictive locomotion, but that it did not play a necessary role in patterning and coordinating the activity of various muscles between segments. Because patterns occurred in the absence of sensory feedback, the hyperthermia-induced failure of pattern generation was not the result of a loss of sensory function at high temperatures.

Application of the neurotransmitter glutamate directly to the muscle allowed an assessment of the thermotolerance of postsynaptic glutamate receptor function. Glutamatergic receptors were still functional at temperatures that were nonpermissive to motor circuit function. This suggests that thermotolerance of behavior was not limited by the thermosensitivity of neuromuscular glutamate receptors because the muscle was still able to be excited.

Explanations for hyperthermia-induced failure of circuit function include the interruption of axonal conduction and the disruption of vesicle release. Mean quantal content (amount of stimulus-induced transmitter release) has been shown to diminish with increasing temperature (Karunanithi et al. 1999). After HS this temperature-induced reduction in quantal content is attenuated, thus protecting the release of transmitter. Reductions in the efficiency of release mechanisms to synchronize large numbers of vesicles at the appropriate times during calcium-mediated exocytosis could explain the reduction in peak intraburst frequency observed at high temperatures. Numerous pathways involved in calcium handling are affected by both HS and heat shock proteins (Barclay and Robertson 2003; Kiang et al. 1992, 1998) and recently it has been demonstrated that Hsp70 can interact with and prevent the thermal inactivation of a Ca^{2+}-ATPase in skeletal muscle (SERCA1a; Tupling et al. 2004). Disruptions in synchronizing transmitter release can result when calcium handling is disrupted (Chuhma and Ohmori 2002). The constitutively expressed version of Hsp70, referred to as Hsc70 (heat shock cognate 70), is part of a trimeric complex with small glutamine-rich tetratricopeptide repeat domain protein (SGT), and cysteine string protein (CSP), which acts as an ATP-dependent chaperone (Tobaben et al. 2001). This complex is functionally involved in the

uncoating of clathrin-coated vesicles, and the regulation of SNARE complex–associated protein (Zinsmaier and Bronk 2001). Disruption of CSP, involved in calcium-activated transmitter release, results in a reduction of thermotolerance of neuromuscular transmission (Zinsmaier et al. 1994) and a significant increase in asynchronous evoked release (Heckmann et al. 1997). Hsc70 interacts with CSP and therefore it is tempting to hypothesize that hyperthermia-induced failure of motor pattern generation might begin with the denaturing of proteins involved in calcium-activated transmitter exocytosis that can be mitigated by moderate levels of Hsp70. Further investigations of calcium handling and evoked release could clarify how hyperthermia disrupts motor function and how this can be protected by HS pretreatment.

HS-induced protection in Canton-S larvae was evident as an ability to travel faster and farther at high temperatures (see above). Thus transient overexpression of Hsp70 after HS of *traII* larvae did not confer enhanced protection of behavior or circuit output as expected. Instead, a decrease in performance of larvae with the highest levels of Hsp70 was observed for some parameters of locomotion, suggesting that heat shock–mediated ubiquitous overexpression of Hsp70 is deleterious. Chronic overexpression of Hsp70 decreases longevity of flies (Krebs and Feder 1997) and disrupts wing morphology, walking speed, and flight performance (Roberts et al. 2003), but only in organisms reared at elevated temperatures. Nevertheless, enhanced thermotolerance of larval neuromuscular transmission conferred by heat shock–induced Hsp70 overexpression has been observed (Karunanithi et al. 2002). In previous studies, only flies reared under stressed, suboptimal conditions have shown enhanced protection of behavior resulting from Hsp70 overexpression (e.g., for flight performance; Roberts et al. 2003). The Karunanithi et al. (2002) study is consistent with the use of disadvantaged *Drosophila*, although it is also possible that experimental design differences with our study could be responsible for the difference in results. Bearing in mind that the previous study examined protection at the neuromuscular junction, whereas the present investigation examined protection of central pattern generation and locomotion, differing expression patterns for Hsp70 could be responsible for the disparity. Although overexpression of Hsp70 at the neuromuscular junction may be protective for synaptic transmission, ubiquitous overexpression of Hsp70 may be deleterious to the whole organism, perhaps because of a diversion of necessary resources in other regions. Alternatively, the beneficial effects of extra copies of Hsp70 may be manifest only when there is a significant temporal component to the stress (i.e., a more sustained stress). At high temperatures, temporal factors do play a role in determining the stress dose or extent of damage applied to the system and in determining the onset and manifestation of the protective mechanisms (Klose et al. 2004). Thus extra Hsp70 may not have been sufficient to improve on the protective effects conferred by normal levels of Hsp70 during our more acute temperature ramps. This explanation would be consistent with the role of Hsp70 as a chaperone requiring time to offset the damage associated with the accumulation of denatured proteins.

Our study does not exclude the involvement of Hsp70 in HS-mediated thermoprotection of locomotion, although it does show that Hsp70 is not a limiting factor. Other candidate proteins for conferring HS-mediated thermoprotection do exist because numerous members of the heat shock protein family are upregulated during stress when most other proteins are downregulated (Parsell et al. 1993). A number of Hsps interact with elements of the cytoskeleton, which is one of the earliest and most sensitive cellular targets of stress (Dalle-Donne et al. 2001). Recent findings have shown that cytoskeletal elements are involved in thermoprotection of locust neuromuscular transmission (Klose et al. 2004). The cytoskeleton can contribute to activity-dependent processes underlying synaptic plasticity through regulation of cellular morphology and signaling proteins (Molitoris 1997). Hsp 90, which interacts with microtubules, as well as Hsp 27 and α-crystallin, which interact with microfilaments (Liang and MacRae 1997), could confer stress-induced thermoprotection. Interestingly, overexpression of Hsp 27 has recently been shown to increase longevity in *Drosophila* (Wang et al. 2004).

Prior heat shock confers protection of larval behavior at least in part by protecting the output of neural circuitry responsible for locomotion. The involvement of Hsp70 in heat shock–mediated protection remains unclear, although it is clear that ubiquitous overexpression of Hsp70 after HS using the *traII* line does not confer enhanced thermoprotection of the locomotor circuit, and may in fact be deleterious during acute hyperthermic bouts of stress. Future studies involving the tissue-specific induction of Hsp70 using the GAL4-UAS system will help to clarify the protective role of Hsp70.

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