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ORIGINAL PAPER

Tissue-specific targeting of Hsp26 has no effect on heat resistance of neural function in larval *Drosophila*

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Abstract Hsp26 belongs to the small heat-shock protein family and is normally expressed in all cells during heat stress. We aimed to determine if overexpression of this protein protects behavior and neural function in *Drosophila melanogaster* during heat stress, as has previously been shown for Hsp70. We used the UAS-GAL4 expression system to drive expression of Hsp26 in the whole animal (ubiquitously), in the motoneurons, and in the muscles of wandering third-instar larvae. There were slight increases in time to crawling failure and normalized excitatory junction potential (EJP) area for some of the transgenic lines, but these were not consistent. In addition, Hsp26 had no effect on the temperature at failure of EJPs, normalized EJP peak amplitude, and normalized EJP half-width. Overexpression larvae had a similar number of motoneuronal boutons and length of nerve terminals as controls, indicating that the occasional protective effects on locomotion were not due to changes at the synapse. We conclude that overexpression had a small thermoprotective effect on locomotion and no effect on neural function. As it has been shown that Hsp26 requires action of other Hsps to reactivate the denatured proteins to which it binds, we propose that at least in larvae, the function of Hsp26 was masked in the relative absence of other Hsps.

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Introduction

Cells express numerous heat-shock proteins (Hsps) during hyperthermia, and there is much evidence establishing a direct involvement of Hsps in thermoprotection. One important result of heat stress is the impairment of neural circuitry, which can cause failure of essential motor pattern generators (Robertson 2004). Thus, it is likely that evolutionary mechanisms have arisen to protect such essential neural circuits, and one important task in this field is the determination of the number and types of Hsps involved and their possible mechanisms of action. One approach to studying this question is to target expression of certain candidate Hsps to tissues of interest.

Hsp70 overexpression protects presynaptic and postsynaptic function in *Drosophila* by reducing the failure rate of neurotransmitter release and stabilizing the amplitude of quantal currents, respectively (Karunanithi et al. 1999). Similar research shows that *Drosophila* larvae overexpressing Hsp70 in motoneurons crawl longer than controls when placed on a hot substrate (Xiao et al. 2007), but that ubiquitous Hsp70 overexpression may be deleterious (Klose et al. 2005), which indicates that Hsp70 is essential specifically in motoneurons. In mammalian neuronal cells, synaptic parameters can be protected by both thermal preconditioning and incubation with Hsp70 (Kelty et al. 2002), and cell death is delayed by a combination of Hsp27 and Hsp70 (Patel et al. 2005).

While Hsp70 seems to be a key player in thermoprotection, the family of small Hsps (sHsps) may also have important roles. sHsps exist as large oligomers during normal physiological conditions and dissociate into active subspecies at high temperatures (Haslbeck et al. 1999). When dissociated, sHsps subspecies bind to denatured

proteins to form large complexes (Stromer et al. 2003) and prevent the irreversible aggregation of these substrate proteins by holding them in a reactivation-ready state (Haslbeck et al. 1999). It appears, however, that sHsps require other Hsps to refold and reactivate the bound proteins (Haslbeck et al. 1999; Lee and Vierling 2000; Stromer 2003), suggesting that their full function is not carried out in the absence of other proteins.

There are four main sHsps in *Drosophila*—Hsp22, Hsp23, Hsp26, and Hsp27—and overexpression of each of these in *Drosophila* is associated with increased lifespan and stress resistance (Wang et al. 2004; Morrow et al. 2004; Morrow et al. 2006). We wanted to determine whether this thermoprotection is evident at the level of neural circuits, and so we examined the neuromuscular junction (NMJ) in *Drosophila* larvae overexpressing Hsp26.

We chose to study the effects of Hsp26 overexpression in *Drosophila* because Hsp26 is localized to the cytosol and appears to associate with the cytoskeleton (Leicht et al. 1986). We have previously shown that the cytoskeleton is important for neural thermotolerance in the locust *Locusta migratoria* (Klose et al. 2004; Garlick and Robertson 2007), which may involve the action of sHsps as chaperones for actin filaments in the cytoskeleton during stress (Mounier and Arrigo 2002). We took advantage of the UAS/GAL4 overexpression system and examined several *Drosophila* Hsp26 overexpression genotypes during heat stress, and assessed crawling behavior and parameters previously linked with synapse function and stability, including morphology (Stewart et al. 1996; DiAntonio et al. 1999; Wan et al. 2000), presynaptic integrity (Karunanithi et al. 1999), and excitatory junction potential (EJP) responses in the muscle (Karunanithi et al. 2002; Newman et al. 2005). We expected that larvae overexpressing Hsp26 would exhibit conserved synaptic function at higher temperatures, that nerve firing and EJPs would fail at higher temperatures, and that EJP peak amplitude, area, and half-width would be greater than in controls during hyperthermia.

Materials and methods

Transgenic flies

The w1118, the w[*]; P{w[+mW.hs]=GawB}how[24B] (Brand and Perrimon 1993) and the w[*]; P{w[+mW.hs]= GAL4-da.G32}UH1 GAL4 enhancer-trap strains were obtained from the Bloomington *Drosophila* stock center. The w[*]; P{w[+mW.hs]=GawB}D42 GAL4 enhancer-trap strain was provided by G. Boulianne (Parkes et al. 1998). The OK6 GAL4 enhancer-trap was provided by K. Dawson-Scully (Aberle et al. 2002). The w[1118]; P{w[+mC]=UAS-hsp26}II and w[1118]; P{w[+mC]=UAS-hsp26}III UAS-hsp26 strains were obtained from S. Benzer (Wang et al. 2004). Flies overexpressing hsp26 were obtained by crossing females carrying the UAS-hsp26 construct with males carrying a GAL4 transgene. To exclude effects due to heterosis, two controls were used: a UAS-alone control and a GAL4-alone control obtained by crossing, respectively, the UAS strain and GAL4 strain with w1118. Flies were raised on standard medium (0.01% molasses, 8.2% cornmeal, 3.4% killed yeast, 0.94% agar, 0.18% benzoic acid, 0.66% propionic acid) at 25°C.

Western blot

Western immunoblotting was used to test overexpression of Hsp26 only in da-GAL4 genotypes, using a previously described protocol (see Xiao et al. 2007). Transgenic Hsp26 was recognized with primary mouse anti-Hsp26 antibody (from Dr. Robert Tanguay, Université Laval) at 1:200 and secondary horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (170-6516, Bio-Rad) at 1:5,000. α-Tubulin was used as an internal loading control and was recognized by anti-α-Tubulin (ab7291, Abcam) at 1:5,000.

Immunostaining

Third-instar larvae were dissected and fixed in 4% paraformaldehyde (diluted in 1× phosphate buffered saline (PBS, Sigma-Aldrich)) for 30 min. For visualization of tissue-specific Hsp26 expression, larvae were rinsed with PBTX [PBS + 0.1% Triton X-100 (Sigma-Aldrich) + 0.1% Bovine Serum Albumin (BSA, Sigma-Aldrich)], washed in PBTX for 30 min, then incubated in a 1:25 dilution of mouse Hsp26 primary antibody (from Dr. R Tanguay, Université Laval) for 2 h at room temperature. The larvae were rinsed and washed in PBTX again (30 min), then incubated in a 1:100 dilution of Cy3 conjugated mouse IgG secondary antibody (Cat no. ab6945, Abcam) for 1 h at room temperature. We stained three larvae from each genotype.

For visualization of synapse morphology, larvae were rinsed, incubated in PBTX for 1 h, then incubated in a 1:50 dilution of Cy3 conjugated anti-HRP antibody (Cat no. 123-165-021, Jackson ImmunoResearch) at 4°C overnight.

Slides were prepared using 50% glycerol in PBTX. Fluorescence was visualized on an Axioplan 2 imaging universal microscope system (ZEISS, NY), and snapshot images were acquired with Openlab 4.0.1 (Improvision, MA). Lengths of terminal arbors and numbers of boutons in muscle 6/7 at abdominal segment 2/3 were visualized and measured using Adobe Photoshop 7.0 (Adobe Systems Inc.). Terminal arbors were defined as the distal ends of the branching motoneuronal axon branching onto the muscles.

Boutons were resolved as spherical bodies, with diameters at least 1.5 times larger than that of the nerve fiber (Cullheim and Kellerth 1978). We analyzed between 9 and 12 larvae for each genotype.

Dissections

Third-instar larvae in the wandering phase were used for all dissections and experiments. Larvae were placed in a dissection dish with hemolymph-like solution HL3 (Stewart et al. 1994) or HL6 (Macleod et al. 2002). It is suggested that HL6 provides better stability for *Drosophila* NMJ preparations (Macleod et al. 2002), and for this reason, it was used for all EJP recordings. However, a subsequent analysis showed that preparations perfused with HL6 showed no difference in EJP failure from preparations perfused with HL3, so HL3 was used for the measurements of evoked nerve activity.

Larvae were immobilized with two magnetic pins onto the head and tail ends of the animal, and an incision was made on the dorsal midline. The viscera were removed, leaving only muscles, cuticle, and nervous system. For EJP recordings, the central nervous system was also removed by cutting all nerve roots. A stimulation suction electrode was placed on the distal cut end of a selected nerve.

Locomotory behavior

An aluminum plate covered with a thin layer of 1% agar was divided into seven circular partitions (9 mm diameter). Eight to ten larvae were placed in each subdivision, allowing for up to seven genotypes to be monitored concurrently. Five of the seven partitions were used during recordings, to house two GAL4 + UAS lines, two UAS controls, and a GAL4 driver control. For example, the D42 trials included D42 + w (driver control), UASII + w and UASIII + w (UAS controls for each insertion point), and D42 + UASII and D42 + UASIII.

The plate was placed in a water bath so that agar temperature could be maintained at 40°C. For the first 20 trials, agar temperature was taken immediately after the recording to ensure consistency within and between recordings. A Logitech webcam was placed directly above the plate, and time-lapse recordings (one frame per second) were acquired using Logitech ImageStudio 7.3. Recordings were analyzed using VideoPoint 2.0 (Lenox Softworks, MA). Crawling failure was defined as a cessation of forward or backward movement across the agar. Time to crawling failure was calculated for each larva, then averaged across all larvae in a partition. The plate was rotated clockwise by 60° between experiments to minimize potential placement effects. After recordings, agar plates were allowed to cool for at least an hour.

Intracellular recordings

Excitatory junction potentials (EJPs) were recorded from the NMJ of muscle 6 in segments A2/3. Larvae were dissected and pinned in hemolymph-like solution, HL6. The CNS was removed. Recordings were made using glass microelectrodes filled with 3 M KAC (40–60 MΩ). Suprathreshold nerve stimulation was achieved with a saline-filled suction electrode and a GRASS S88 stimulator (GRASS Instruments, USA; pulse duration: 2 ms; pulse frequency: 0.2 pps). Steady saline flow was maintained using a Peri-Star pump, and temperature was elevated using a PTC03 heater (P.E. Scientific Systems and Design). A thermocouple placed near the head of the larva monitored saline temperature. Signals were amplified by a Neuroprobe Amplifier 1600 (A-M Systems, Inc., WA) and digitized with a Digidata 1322A (Axon Instruments, Foster City, CA). Signals were acquired and analyzed with pClamp 9.2 software (Axon Instruments, Foster City, CA).

Between 10 and 15 animals were used for each genotype. All drivers were used, as well as all appropriate controls (14 genotypes). The temperature was ramped at approximately 5°C/min, allowing a 1- to 2-min plateau at 36°C. After this plateau, temperature elevation was resumed at 5°C/min until EJP failure (42–45°C).

Extracellular recordings

Larvae were dissected as described above, but with the CNS, brain lobes, ventral ganglia, and segmental nerves left intact. Dissected larvae were pinned in HL3 solution. Extracellular suction electrodes were used to record evoked activity in nerves innervating segments A6/7. Saline contained 10^{-4} mM pilocarpine (Sigma-Aldrich) to induce spontaneous activity of the nerves. Pilocarpine is a cholinergic agonist previously shown to induce spontaneous activity in a *Drosophila* central pattern generator (CPG) (Gorczyca et al. 1991). Recordings were amplified with a P15 Amplifier (GRASS instruments) and digitized with a Digidata 1322A (Axon Instruments, Foster City, CA). Signals were analyzed with pClamp 9.2 software (Axon Instruments, Foster City, CA).

Between seven and ten larvae were used for each genotype. Only the da-GAL4 driver was used, as well as the appropriate controls (five genotypes). The temperature was elevated at 5°C/min past failure of spontaneous activity (36.0±3.6°C) until saline temperature reached 42–45°C.

Statistics

The following variables were recorded and compared between groups to assess differences in thermotolerance between genotypes: time to crawling failure (locomotory

behavior); temperature at failure, peak amplitude, half-width, and area of EJPs (postsynaptic integrity); temperature at failure of spontaneous nerve activity (presynaptic integrity); and bouton number and length of terminal arbors (synaptic morphology). EJP peak amplitude, half-width, and area were analyzed at the following temperature intervals: 25°C, 30°C, 35°C, 40°C, 42°C, 45°C (data at 45°C were later excluded, as few animals produced EJPs at this temperature and sample sizes were insufficient for statistical analysis). Data were normalized by equating values at 25°C to one and calculating all other values relative to these.

All statistical analyses were performed with JMP IN 5.1 (SAS Institute). Differences between groups were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's HSD (honest significant difference) post hoc test for comparison of multiple means. For analysis of normalized EJP parameters (peak amplitude, half-width, and area), data were fitted to ANOVA models with repeated measures. Post hoc contrasts were used to compare each overexpression mutant (OM) genotype with its two controls when the models indicated significant between-group differences, and later Tukey's HSD analyses were used to determine at which temperatures the significant differences were found. Significance was established if each GAL4 + UAS genotype was significantly different from both the UAS and the GAL4 controls. Significance was assessed at $\alpha = 0.05$. An asterisk is used in figures to indicate significance.

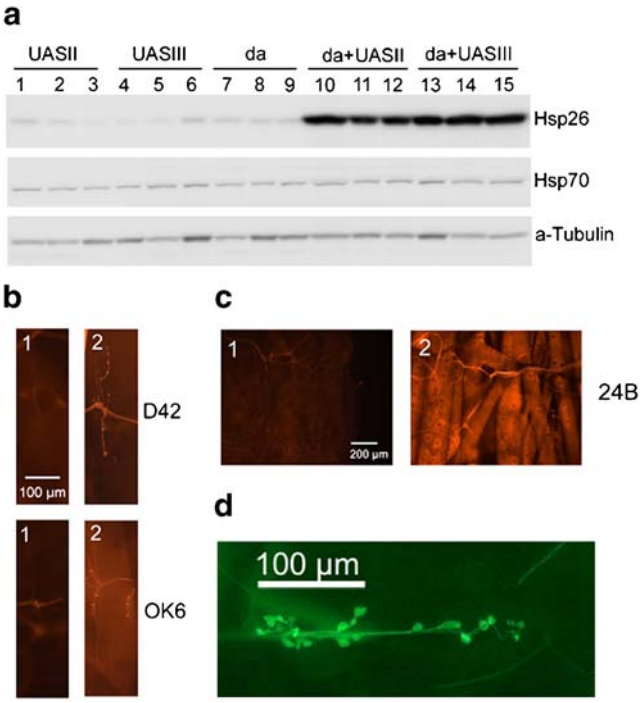

Fig. 1 Expression of transgenic Hsp26 under control of different tissue-specific drivers. **a** Western immunoblot of whole body lysates shows higher levels of ubiquitous Hsp26 expression in da-GAL4 + UAS hsp26 larval genotypes (*lanes 10–15*) compared with control genotypes (*lanes 1–9*). Three vials of ten larvae were used for each genotype. *UASII and UASIII* are controls for insertions on chromosomes two and three, respectively; *da* is the control for the ubiquitous driver da-GAL4; *da + UASII* and *da + UASIII* are strains overexpressing *hsp26* on chromosomes two and three, respectively. *α-Tubulin and Hsp70* were used as loading controls and show no difference in expression between the groups. **b** Immunofluorescence in terminal axonal boutons from abdominal segments in D42 and OK6 transgenics (*panel 2*; D42 + UASIII and OK6 + UASIII shown) but not in the respective controls (*panel 1*; D42 alone and OK6 alone shown as controls). **c** Abdominal muscles are stained in 24B transgenic larvae (*panel 2*; 24B + UASIII shown), but not in controls (*panel 1*; 24B alone shown as control). Immunostaining for **b** and **c** was achieved with Hsp26 primary antibody and Cy3-conjugated secondary antibody on whole mount third-instar larval dissections. A single representative sample is shown for each. **d** Sample image of HRP immunostaining of the terminal arbor of muscle 6/7 used for determining bouton number and arbor length

Results

Ubiquitous overexpression

Western blotting was performed using ubiquitously expressing larvae to confirm that Hsp26 was overexpressed in these genotypes (da + UASII and da + UASIII). Three replicates were used per genotype, and both transgenic lines clearly show higher levels of Hsp26 expression than controls, signifying overexpression in both larval genotypes—those containing the UAS-Hsp26 insert on chromosome II and those containing the insert on chromosome III (Fig. 1a). There were no differences in expression levels between the two insertion points. Hsp70 and α-tubulin were used as loading controls, as they are not expected to be overexpressed at room temperature in any of the genotypes.

Muscle and motoneuron overexpression

To confirm tissue-specific overexpression of Hsp26 with the GAL4 drivers D42 (motoneuron), OK6 (motoneuron), and 24B (muscle), we performed immunohistochemistry on three larvae of each genotype. Larvae were fixed and incubated in primary Hsp26 antibody, then incubated in secondary antibody conjugated with Cy3 (fluorescent protein). Motoneuronal OM (D42 + UASII, D42 + UASIII, OK6 + UASII, and OK6 + UASIII) differed from controls at the NMJ. Controls had no fluorescence in the boutons, indicating a lack of Hsp26 expression (Fig. 1b). On the other hand, there were well-defined fluorescent boutons at the NMJ in OM, confirming that Hsp26 levels were higher at the synapse in these mutants.

Muscle OM (24B + UASII and 24B + UASIII) were also clearly different from controls (Fig. 1c). Muscles were

brightly labeled, indicating Hsp26 overexpression was strong and uniform. Muscle cell structure could be resolved, and nuclei appeared as dark spots along the muscle fiber.

No apparent difference in Hsp26 overexpression between inserts on chromosome II and chromosome III could be detected (data not shown), which is consistent with the western results. The differences in specific labeling of tissues between OMs and the controls were clear, and consequently, we did not attempt to quantify exact levels of Hsp26 overexpression.

Chronic overexpression of Hsp26 does not obviously change synapse morphology

The GAL4 + UAS system forces overexpression of Hsp26 throughout all stages of development; this chronic constitutive expression is different from the expression patterns in control animals and may lead to permanent morphological and functional changes at the synapse. To confirm that Hsp26 overexpression in the transgenic larvae did not affect morphology, we performed immunostaining of whole-mount dissections from all the genotypes using a fluorescent HRP antibody. Images of synapses were taken at 200× magnification, high enough to visualize and count boutons, and measure lengths of terminal arbors (Fig. 1d). Type Is and Ib boutons were counted together. There were no differences between groups in bouton number or length of arbors ($p>0.05$, one-way ANOVA with Tukey's HSD; Table 1). Chronic overexpression of Hsp26 throughout embryonic and early larval stages had no effect on the gross morphology of the nerve terminal. Boutons were not viewed at higher magnification to visualize the distribution of neurotransmitter release machinery.

Hsp26 overexpression has inconsistent effects on locomotion

Locomotion activity during heat shock was examined by allowing larvae on agar plates to rest on the surface of a hot water bath. It was expected that genotypes overexpressing Hsp26 would show increased resistance to heat and would crawl for longer periods than the controls.

Larvae in all trials stopped crawling between 17.4± 0.5 min and 34.4±0.9 min after being placed in the water bath. Maximum observed agar temperature was 41.8°C, average temperature was 40.6°C, and minimum temperature was 39.9°C.

Some larvae continued twitching for some time after crawling failure. This was a variable behavior, and could last for the remainder of a recording, particularly if larvae were shielded from the agar by climbing on each other. For this reason, twitching behavior was not regarded as crawling activity and was not used in calculating time to crawling failure. During the cooling period at the end of the recordings (>1 h), there was no recovery of locomotory behavior or other movements, suggesting all larvae had been killed.

da/UASII larvae (ubiquitous expression) crawled significantly longer than controls during heat shock ($p<0.05$, one-way ANOVA with Tukey's HSD; Fig. 2). da/UASIII larvae appeared to have greater time to crawling failure than controls, but this was not statistically significant (HSD=−21.6, $q=2.7$). The motoneuronal drivers differed in their effects: OK6 + UASIII (but not OK6 + UASII) showed increased time to crawling failure ($p<0.05$, one-way ANOVA with Tukey's HSD), whereas D42 larvae showed no difference compared with controls ($p>0.05$;

Table 1 Synaptic characteristics and temperature at failure of spontaneous nerve activity

Group	Number of boutons (*N*)	Length of terminal arbor	Failure temperature[a] (°C) of spontaneous nerve activity (*N*)
UASII	32.0±1.3 (6)	253.6 (7)	37.1±2.5 (10)
UASIII	37.9±1.7 (10)	292.5 (10)	34.9±2.0 (8)
da	42.3±3.5 (9)	309.5 (9)	36.9±4.6 (10)
da + UASII	39.7±3.8 (6)	305.9 (8)	36.1±3.0 (7)
da + UASIII	49.9±3.6 (7)	335.7 (7)	36.4±2.7 (9)
D42	52.2±5.7 (7)	346.2 (10)	
D42 + UASII	39.8±1.7 (10)	319.5 (10)	
D42 + UASIII	40.3±3.0 (8)	306.9 (8)	
OK6	63.5±2.8 (10)	346.2 (10)	
OK6 + UASII	44.3±2.0 (10)	302.0 (10)	
OK6 + UASIII	44.8±3.4 (6)	343.6 (7)	
24B	31.2±2.1 (9)	266.2 (9)	
24B + UASII	42.8±4.2 (8)	254.6 (8)	
24B + UASIII	43.4±1.9 (8)	310.6 (8)	

[a] Temperature at failure of nerve activity was only tested in da-GAL4

N=number of recordings; Values are means±SEM

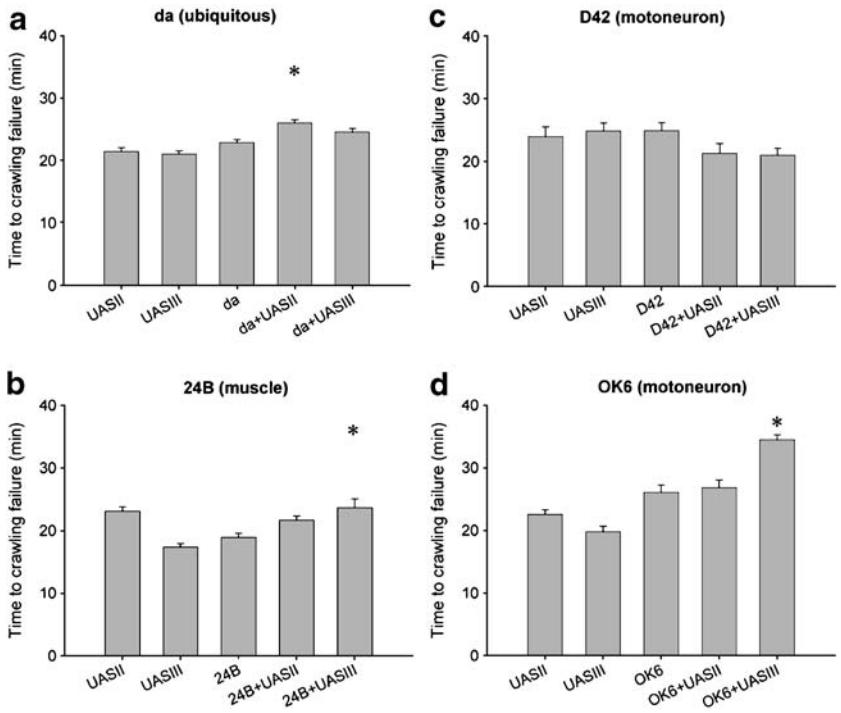

Fig. 2 Time to crawling failure is not consistently affected by Hsp26 overexpression in four groups of larvae with tissue-specific overexpression ubiquitously (**a**), in the muscles (**b**) and in the motoneurons (**c**) and (**d**). Three of these groups showed some difference between the transgenic and control larvae (**a**, **b**, and **d**), but for only one of the two transgenic groups (*$p<0.05$)

Fig. 2). 24B + UASIII larvae (muscle overexpression) crawled longer than controls ($p<0.05$, one-way ANOVA with Tukey's HSD; Fig. 2). These data suggest that there was a small thermoprotective function of Hsp26 in muscles and motoneurons during heat shock.

There were differences in the UASII and UASIII controls between analyses. For example, UASII time to crawling failure ranges from 17.4 min (24B trials) to 24.4 min (D42 trials), which is greater than the difference between 24B + UASIII and UASIII (24B trials), a difference that is statistically significant.

Spontaneous activity in locomotory motoneurons is unchanged

Intact *Drosophila* nerves show spontaneous patterned or bursting activity, presumably originating from the locomotor central pattern generator (CPG). Cessation of activity during hyperthermia implies loss of function, either at the CPG or downstream, in the nerve fibers. Alternatively, cessation of activity may represent a protective switch-off mechanism. To assess the stability of this system in Hsp26 OM during heat shock, we induced increases in spontaneous nerve firing using pilocarpine, a cholinergic agonist.

The quality of recorded activity was variable. Some larvae showed clear patterned activity, whereas others showed arrhythmic bursts, which made it difficult to assess exactly when failure had occurred. These differences were unrelated to genotype. In addition to differences between animals, there were methodological difficulties with signal acquisition. Electrodes were of particular importance because minute differences in electrode diameter at the tip gave rise to large differences in the signal to noise ratio (SNR). In addition, the high amplification of the acquisition signal resulted in low SNR even with an optimal electrode. In some animals, SNRs were so low that the recordings were discarded. A sample of an acquired trace is shown in Fig. 3a.

Temperature was elevated beyond failure of the nerve activity, to about 45°C, and then lowered to room temperature, 25°C. In a few cases, low amplitude, patternless activity was observed during cooling and was assumed to represent some form of recovery. Temperatures at the recovery events were normally higher than failure temperatures. These data were excluded from the analysis because they were rare and inconsistent.

There were no differences between groups in temperature at failure of spontaneous nerve firing ($p>0.05$, one-way ANOVA with Tukey's HSD; Table 1). Mean (±SEM) temperature at failure or nerve activity was 36.0±0.5°C.

EJP parameters are unchanged

Motoneuronal stimulation evokes depolarizations of the muscle membrane (EJPs). To assess functional integrity of the NMJ during heat shock, electrical stimulation was

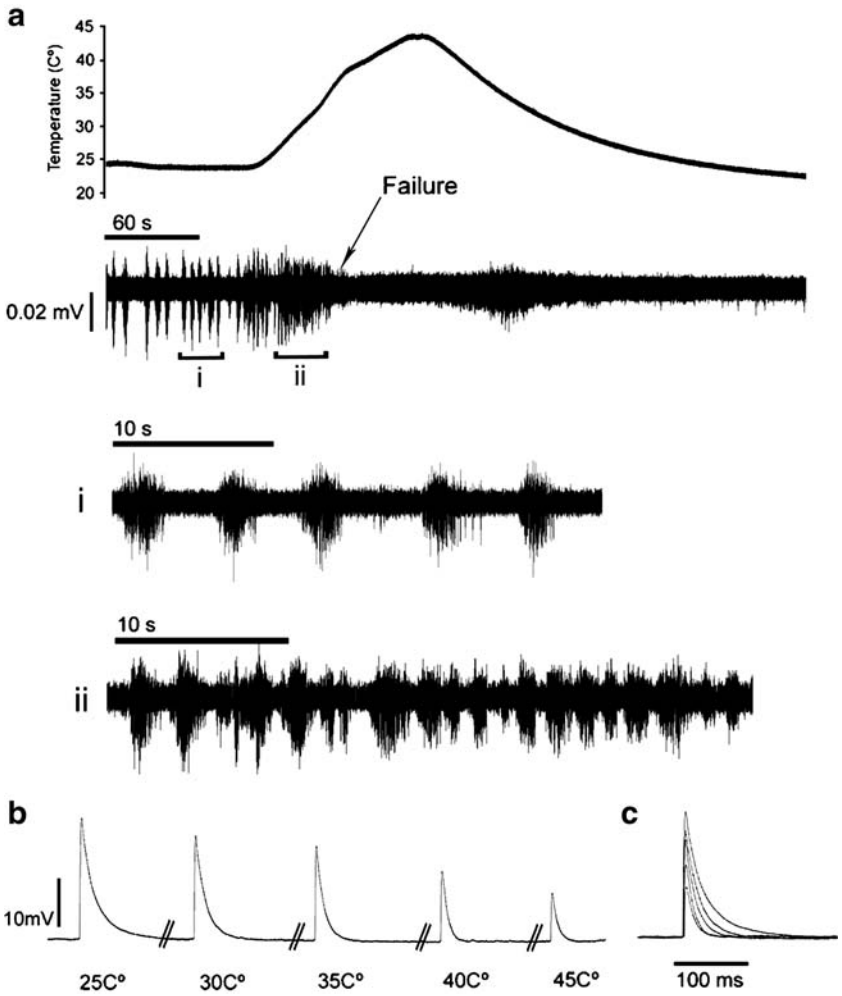

Fig. 3 Extracellular (**a**) and intracellular (**b**, **c**) recordings during temperature ramps. As temperature increases, nerve bursting frequency increases (**a**, *i*) and eventually stops (**a**, *ii*). EJPs in the muscle show decreases in amplitude (**b**) and duration (**c**). No differences were found in the temperature at failure between transgenic and control groups

applied to the nerves with an extracellular suction electrode, and EJPs were recorded with an intracellular electrode. As temperature increased, EJP amplitude normally decreased gradually, until it became negligible (failure; Fig. 3b–c). When saline temperature was decreased, EJPs sometimes resumed, and EJP amplitude gradually increased (recovery). In a few cases, failure and recovery were abrupt. Larvae with abrupt EJP recovery also exhibited a return to near-maximal peak amplitudes (relative to pre-failure).

There were no significant differences between transgenic larvae and controls in time to EJP failure, normalized peak EJP amplitude and normalized EJP half-width ($p>0.05$, one-way ANOVA with Tukey's HSD; Table 2). Normalized EJP area was greater only in da + UASIII transgenics when compared with controls, and only at 35°C and 40°C ($p<0.05$, one-way ANOVA with repeated measures followed by contrasts and Tukey's HSD; Table 2).

Discussion

The small heat-shock proteins (sHsps) are interesting because they seem to operate by a different mechanism from the commonly studied Hsps (e.g., Hsp70), and because although divergent in structure, they have been found whenever they have been sought in all cells in all living organisms. The low levels of constitutively expressed sHsps during normal physiological conditions in conjunction with the fact that sHsps are among the most strongly expressed proteins during heat shock (Haslbeck 2002) implies that their main function is performed during heat stress. Our goal was to examine the involvement of Hsp26 in thermoprotection of neural function. Neural failure can quickly lead to death, especially if thermotaxis is hindered, and protecting neural circuitry during hyperthermia is crucial. Our findings in larval *Drosophila* indicate that

Table 2 EJP failure temperature and normalized EJP size parameters

Group	Failure temp[a] (C°)	Peak amplitude[b]				Half-width[b]				Area[b]			
		30°C	35°C	40°C	42°C	30°C	35°C	40°C	42°C	30°C	35°C	40°C	42°C
UASII	44.9	0.87	0.87	0.81	0.66	0.66	0.54	0.46	0.49	0.55	0.46	0.38	0.36
UASIII	44.4	0.80	0.77	0.69	0.60	0.71	0.56	0.50	0.58	0.52	0.40	0.31	0.27
da	44.1	0.84	0.85	0.81	0.61	0.63	0.57	0.45	0.37	0.55	0.45	0.30	0.28
da + UASII	44.5	0.96	0.82	0.70	0.52	0.78	0.56	0.58	0.48	0.73	0.48	0.39	0.30
da + UASIII	45.5	0.94	0.94	0.86	0.65	0.76	0.69	0.64	0.64	0.70	0.63*	0.59*	0.42
D42	44.3	0.85	0.94	0.78	0.61	0.71	0.67	0.63	0.62	0.59	0.62	0.54	0.48
D42 + UASII	45.1	0.94	0.90	0.89	0.63	0.75	0.64	0.56	0.59	0.62	0.49	0.55	0.38
D42 + UASIII	44.5	0.92	0.91	0.77	0.58	0.65	0.58	0.54	0.44	0.59	0.55	0.46	0.30
OK6	44.8	0.91	0.90	0.80	0.71	0.68	0.53	0.50	0.53	0.57	0.44	0.37	0.33
OK6 + UASII	44.2	0.92	0.86	0.81	0.64	0.76	0.64	0.66	0.56	0.62	0.45	0.41	0.33
OK6 + UASIII	44.4	0.93	0.88	0.79	0.68	0.75	0.62	0.42	0.51	0.65	0.47	0.31	0.31
24B	45.2	0.87	0.87	0.68	0.57	0.81	0.73	0.70	0.75	0.62	0.54	0.40	0.36
24B + UASII	45.5	0.87	0.86	0.83	0.61	0.68	0.55	0.61	0.52	0.52	0.40	0.50	0.35
24B + UASIII	45.0	0.87	0.90	0.89	0.74	0.74	0.66	0.65	0.70	0.57	0.54	0.55	0.47

[a] Failure temperature was recorded as the temperature at which cessation of EJPs occurred
[b] EJP peak amplitude, half-width, and area were obtained for each genotype (between 10 and 15 larvae per genotype) at 25°C, 30°C, 35°C, 40°C, and 42°C and normalized to 25°C
*da + UASIII had significantly greater EJP area at 30°C and 35°C ($p<0.05$) than the respective controls

Hsp26 overexpression does not affect neural function and has negligible effects on crawling behavior during hyperthermia.

We used the GAL4 + UAS expression system to target Hsp26 overexpression to muscles, to motoneurons, and to all tissues (ubiquitously) in *Drosophila* larvae. It has been suggested that GAL4 overexpression during *Drosophila* development can be deleterious and even cause apoptosis (Kramer and Staveley 2003), so we used GAL4 control lines for all analyses to control for these potential deleterious effects. Hsp26 overexpression had no effect on any of the following measures: failure temperature of EJPs, EJP half-width, EJP peak amplitude, bouton number and terminal arbor length at the NMJ, and failure temperature of spontaneous nerve excitation. There were slight increases in EJP area and time to crawling failure in some OM, but the effects were inconsistent, and they were never seen in both OM lines (one with the UAS-hsp26 insert on chromosome two and another with the insert on chromosome three).

Our negative findings are contrary to findings by Wang et al. (2004), but several factors can account for this. For example, we used larvae, and Wang et al. (2004) used adult *Drosophila*. This is unlikely to be of major significance, as Hsp26 mRNA is present in *Drosophila* from early embryonic stage to adulthood (Glaser et al. 1986; Morrow and Tanguay 2003). On the other hand, it may be important if the thermoprotective function of Hsp26 is developmentally regulated, as is the case for Hsp70 in mouse oocytes (Curci et al. 1987).

The slopes of the temperature ramps also differed between the two studies. We rapidly increased temperatures beyond 40°C for all analyses to measure acute hyperthermic stress resistance. Wang et al. (2004) increased temperature to 36°C and held it for 4 h, measuring accumulating effects of prolonged sublethal heat stress. The amounts and types of cellular damage in the two scenarios will differ. A temperature of 36°C is the suggested maximum for physiological studies of heat shock because tissue damage occurs above this temperature (Karunanithi et al. 1999). However, temperatures often exceed 35°C and can reach 45°C in necrotic fruit, *Drosophila*'s ecological niche (Feder et al. 1996). Thus, elevating temperature beyond 40°C was deemed appropriate.

Furthermore, holding temperature at 36°C for hours is likely to stimulate induction of multiple Hsps, some of which have been shown to interact with Hsp26 in refolding denatured proteins (Cashikar et al. 2005; Stromer et al. 2003). In this protocol, Hsp26 may be working in concert with other Hsps to produce the marked thermotolerance effect that was noted by Wang et al. (2004). We chose to increase temperatures rapidly and induce hyperthermia in under 15 min, potentially minimizing the induction and activation of other Hsps.

Locomotion during hyperthermia

Locomotory activity is a behavioral indicator of neuronal function and is particularly relevant during heat stress.

Generally, we found little evidence that locomotion is thermoprotected by Hsp26. Transgenic larvae in three of the four driver groups showed longer crawling duration during hyperthermia compared with controls, but only at one insertion point. Furthermore, the insertion genotypes which showed significant differences from controls differed: in da lines (ubiquitous overexpression) larvae with the UAS-hsp26II insert crawled for longer periods than controls, whereas in 24B (muscle overexpression) and OK6 (motoneuron overexpression) lines, larvae with the UAS-hsp26III crawled longer. The D42 (less specific motoneuron driver) lines showed no increase in crawling duration for both UAS insertion lines. To be certain of an effect, we expected both transgenic lines for each driver to show significant effects.

There were also differences between UASII and UASIII controls in this analysis because UASII and UASIII lines were analyzed numerous times for each driver. Pooling UASII values and pooling UASIII values from all recordings would have provided a larger sample size with which to compare OM, but possible non-sampling effects (e.g., rearing differences) may have thus been overlooked. On the whole, these inconsistencies attest to the marginality of the effects of Hsp26 overexpression on thermal resistance.

Synapse morphology in mutants

Hsp26 is constitutively expressed in numerous tissues (including the CNS, epithelium, and spermatocytes) during development in *Drosophila* (Glaser et al. 1986) and expression peaks during late third-instar or early pupal stage (Sirotkin and Davidson 1981; Glaser et al. 1986). Although this suggests that *Drosophila* are well-adapted to background levels of Hsp26, chronic overexpression of another Hsp, Hsp70, is associated with changes in synaptic morphology, such as increases in cumulative bouton area and number of large (Ib) boutons (Xiao et al. 2007). However, modifications in synapse morphology may be compensated. This was shown in a FasII *Drosophila* mutant that has significantly lower levels of Fascilin II (a cell adhesion molecule) than wild type, resulting in greatly reduced bouton numbers but normal synaptic function (Stewart et al. 1996).

We may have overlooked some morphological changes by combining boutons Ib and Is. Some studies include separate counts of these boutons because type Ib (large) boutons generate less quantal output and result in smaller EJPs than type Is boutons (Kurdyak et al. 1994; Li et al. 2002), and it is possible that Hsp26 has a stronger association with one of these bouton types. However, with no difference in combined numbers, any increase (or decrease) in one type would have to have been offset by a compensatory decrease (or increase) in the other, and we believe this is unlikely.

Motoneuronal activity

To assess whether signaling in motoneurons was protected by Hsp26 overexpression, we used the cholinergic agonist pilocarpine to evoke rhythmical bursting in the CPG and recorded the resulting nerve activity using an extracellular suction electrode. Rhythmic motor patterns in semi-intact larvae have been observed through intracellular recording of muscle potentials (Barclay et al. 2002), but extracellular recording from motoneurons is more challenging, and does not contain information about amplitude and other features of the action potentials. Pilocarpine has been used to evoke patterned motor output in other insects, including moths (Johnston and Levine 1996) and locusts (Ryckebusch and Laurent 1993). In *Drosophila,* it has been used to stimulate rhythmic bursting in semi-intact pharyngeal muscle preparations (Gorczyca et al. 1991) but not to induce locomotor patterns. As locomotory motoneurons in *Drosophila* receive chiefly cholinergic input (Rohrbough et al. 2003), we were confident that pilocarpine would sufficiently evoke motoneuronal activity in the segmental nerves.

Our examination of cessation of nerve firing may not have been a strong indicator of CPG output or of ecologically relevant locomotor output during hyperthermia. Nevertheless the lack of any effect of Hsp26 overexpression on this measure was clear.

Synaptic function

EJPs were used as measures of synaptic function because EJP amplitude is dependent on the amount of postsynaptic neurotransmitter release, and the number and conductance of receptors on the postsynaptic membrane (Stewart et al. 1996). However, EJPs are also dependent on temperature, attaining maximal amplitudes at room temperature and exhibiting decreased amplitude at low and high temperatures (Robertson 1993; Dunn and Mercier 2003). Therefore, one of the difficulties with EJP analysis is the untangling of passive temperature-dependent modifications and true, functional adaptations of the EJP response.

Some studies include measurements of mEJP size as an indicator of synapse function (e.g., Karunanithi et al. 2002). mEJPs are normally assumed to be dependent solely on the postsynaptic sensitivity to neurotransmitter, while evoked EJPs depend on both presynaptic and postsynaptic features (Wan et al. 2000). The mEJP/EJP relationship is complex, and it is possible to observe drastic increases in mEJPs without changes in EJPs (Lnenicka and Mellon 1983). Other studies use excitatory junction currents (EJCs)

instead of EJPs (e.g., Stewart et al. 1996) because EJCs are not confounded by variations in postsynaptic input resistance. However, we reasoned that if true differences in synaptic function exist between OM and control larvae during hyperthermia, then these differences would have been evident in measures of EJPs. As all conditions were consistent between groups of larvae, observed differences should be solely associated with functional differences between groups of larvae.

Prior evidence for thermoprotection of EJP parameters comes from a study of desert-adapted *Drosophila,* which maintain greater peak amplitude and area of the EJPs during heat shock than wild-type *Drosophila* (Newman et al. 2005). However, in the current study, neither temperature at failure of EJPs, nor EJP amplitude or EJP half-width was altered in the overexpression groups. Finally, the increase in EJP area that we observed in the ubiquitous UASIII group, but that was unaccompanied by changes in EJP amplitude or half-width, suggests that this is either a chance anomalous result, or the product of smaller, statistically nonsignificant changes in the other two parameters.

Multiple Hsp involvement

There is mounting evidence that sHsps act as chaperones (Morrow et al. 2006), binding to misfolded proteins and holding them in a reactivation-ready state (Haslbeck 2002), whereas other Hsps are responsible for the disaggregation and reactivation of these proteins (Lee and Vierling 2000; Cashikar et al. 2005). In the case of Hsp26, refolding of aggregates is accomplished by three other Hsps, Hsp70, Hsp104, and Hsp40 (Cashikar et al. 2005). In the absence of these other Hsps, overexpression of Hsp26 might result in the trapping of large quantities of denatured proteins without a means for releasing and refolding them. Hence, any thermoprotective functions would be obscured. Moreover, the expression of multiple Hsps is dramatically more neuroprotective than either Hsp70 or Hsp25 alone in a cultured mouse motoneuron model of ALS (Batulan et al. 2006).

If Hsp26 is activated during hyperthermia solely from previously created storage forms of the protein (Haslbeck et al. 1999), then knock-out mutants may be expected to show a more pronounced response to heat stress. However, yeast Hsp26 null mutants do not differ in thermotolerance from controls (Petko and Lindquist 1986), lending support to the hypothesis that Hsp26 function can only be completed in the presence of other Hsps. We speculate that the function of Hsp26 in *Drosophila* OM may appear silent due to the lack of overexpression of other Hsps, which are normally involved in refolding and reactivating proteins bound to Hsp26 subspecies. If this is correct, then null Hsp26 larvae overexpressing Hsp70, for example, will exhibit decreased thermoresistance compared with larvae overexpressing Hsp70 alone.

Summary

We have demonstrated that Hsp26 has a negligible thermoprotective effect on crawling behavior in *Drosophila* larvae and no effect on neural function. The inconsistent effect of Hsp26 overexpression does not necessarily suggest that its function is not important for thermoresistance. *Drosophila* have many Hsps, and the likelihood that any single Hsp has a dominant role in thermoprotection is small. More likely, multiple Hsps interact in a complex and perhaps redundant fashion to protect the animal during heat stress. Constitutive levels of Hsp26 may be adjusted finely to the needs of the organism within the large network of stress proteins, and increases in Hsp26 without increases in other Hsps may have little effect. Future research should address the thermoresistance of knock-out Hsp26 mutants, to determine whether these larvae are viable, and if so, to what extent they are able to tolerate increases in ambient temperature. Previous research has also shown that overexpression of Hsp22 in *Drosophila* motoneurons confers resistance to oxidative stress and increases lifespan (Morrow et al. 2004). It would be interesting to replicate our study with Hsp22 to study neural function more closely in overexpressing *Drosophila.*

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