Targeting HSP70 to Motoneurons Protects Locomotor Activity from Hyperthermia in *Drosophila*

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ABSTRACT: **Heat shock preconditioning can enhance locomotor and synaptic performance during subsequent hyperthermia. The molecular basis underlying this neural phenotypic modification is largely unknown. Here we report that directing the expression of the 70 kDa heat shock protein (HSP70) to motoneurons protected larval locomotor activity of *Drosophila*. Tissue-specific expression showed that motoneurons were critical for developing HSP70-mediated thermoprotection of locomotor activity, whereas peripheral sensory neurons, dopaminergic neurons, serotonergic neurons, and muscle cells alone were insufficient. Targeting HSP70 to motoneurons caused structural plasticity of axonal terminals associated with increased transmitter release at neuromuscular junctions at high temperature. The thermoprotection induced by motoneuronal expression of HSP70 mimicked the protective effect of a prior heat shock (36°C, 1 h; 25°C, 1 h) but the effects of heat shock and motoneuronal expression of HSP70 were not additive. In the absence of heat shock pretreatment, ubiquitously expressed transgenic HSP70 activated the transcription of endogenous *hsp70* genes. These results demonstrate that motoneurons were critical for HSP70-mediated thermoprotection, and that transgenic HSP70 activated the transcription of endogenous *hsp70* in motoneurons with the result that a mix of transgenic and endogenous HSP70 conferred thermoprotection in *Drosophila* larva.** © 2007 Wiley Periodicals, Inc. Develop Neurobiol 67: 438–455, 2007

Keywords: **HSP70; motoneuron; thermoprotection; locomotor activity**

INTRODUCTION

An important physiological strategy enabling organisms to behave appropriately in variable and often extreme environments involves the modification of neural control circuits to cope with abiotic stressors. Long before the demise of neurons under harsh conditions, neural function is compromised. Thus, effective stress protection involves modifications that can compensate for impairment of neural function (Robertson, 2004a,b). Previous studies have demonstrated that a prior sublethal heat exposure causes profound thermoprotective effects on neuronal function associated with behavioral thermotolerance in *Locusta migratoria* (Barclay and Robertson, 2000; Wu et al., 2001; Money et al., 2005) and *Drosophila melanogaster* (Karunanithi et al., 1999; Klose et al., 2005). The heat shock response and its characteristic upregulation of 70 kDa heat shock protein (HSP70) is a likely mechanism for such behavioral thermoprotection.

The chaperoning functions of HSP70 are regarded as the basis of cellular thermotolerance or cytoprotection (Morimoto, 1993; Nollen et al., 1999; Weibezahn et al., 2004; Mayer and Bukau, 2005; Pespeni et al., 2005). In the nervous system, HSP70 can protect against cerebral ischemic injury and can mitigate neurodegenerative disorders (Auluck et al., 2002; Kelly and Yenari, 2002; Giffard and Yenari, 2004; Jin et al., 2004; Muchowski and Wacker, 2005). Previously we examined how neuronal function is affected prior to hyperthermic cell death and found

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that a prior heat shock could sustain synaptic performance via pre- and post-synaptic modifications occurring in parallel with HSP70 induction (Karunanithi et al., 1999). Extreme increases of HSP70 by heat shocking transgenic flies carrying 12 extra copies of an *hsp70* gene (Welte et al., 1993) showed enhanced thermoprotection of presynaptic performance (Karunanithi et al., 2002). In addition, incubation of mouse brainstem slices with inducible HSP70 mitigates the effects of thermal stress on parameters of neural transmission (Kelty et al., 2002).

Increasingly, molecular genetic approaches are being applied to the investigation of neuronal networks to identify the molecular and cellular underpinnings of the neural circuitry controlling behavior (Sokolowski, 2001; Kullander, 2005; Wulff and Wisden, 2005). These approaches have proven to be successful in addressing the peristaltic locomotor pattern of larval *Drosophila* (Barclay et al., 2002; Fox et al., 2006). Recently we showed that heat pretreatment protected larval peristaltic locomotion of the Canton S (CS) strain by attenuating the detrimental effects of hyperthermic stress on locomotor velocity, the frequency of rhythmic peristaltic contractions, and the distance traveled (Klose et al., 2005). In transgenic larvae containing 12 extra copies of *hsp70* genes, however, locomotor activities were not enhanced and appeared mildly impaired compared to their transgenic insertion control (Klose et al., 2005) in spite of the enhanced synaptic performance previously demonstrated (Karunanithi et al., 2002). To clarify the role of HSP70 in neural thermoprotection, we targeted HSP70 ubiquitously, to the muscular system and to elements of the nervous system, and examined the locomotor activity of third-instar larvae at high temperature. Our findings indicate that targeting HSP70 to motoneurons is sufficient to induce thermoprotection of behavior in *Drosophila* larvae.

MATERIALS AND METHODS

Transgenic Flies

The full length *Drosophila hsp70Ab* gene coding sequence (CDS) was rescued from *Xho*I-*Sal*I digested plasmid 56H8 (Schedl et al., 1978). The *hsp70Ab* fragment was inserted into a *Xho*I-*Sal*I opened Gal4-responsive vector pINDY5. Because *Xho*I and *Sal*I generated the same sticky ends of DNA, a new construct with right *hsp70* direction was selected. The new construct pINDY5-hsp70 contained a c-myc tag that encodes 10 amino acid sequence (EQKLISEEDL) following the *hsp70Ab* CDS. The pINDY5-hsp70 was microinjected into 30-min old w1118 embryos. The transformants were then crossed to ubiquitous Gal4 driver (da-Gal4) and the transgenic product at the third-instar larval stage was screened. At least 11 UAS-hsp70 fly lines with independent insertions were established. Two transgenic lines (UAS-hsp70-4.3 and UAS-hsp70-9.1, in short, 4.3 and 9.1) with relatively higher expression were selected for further experiments. The Gal4 driver lines used in this study were ubiquitous driver (da-Gal4), muscle-specific driver (24B-Gal4), motoneuron-specific drivers (D42-Gal4 and OK6-Gal4), sensory neuron-specific driver (P0163-Gal4), and dopaminergic neuron-specific driver (ddc-Gal4). Other strains used are w1118, Canton-S (CS), and UAS-CD8GFP (UAS-GFP). All the flies were raised with standard cornmeal medium at 25°C in an incubator with 60–70% humidity. The third-instar wandering stage larvae were used for the following experiments.

Western Blot

For sample preparation, 10 larvae of each genotype were homogenized in 100 μl of ice-cold extract buffer (10 m*M* Tris•Cl, pH 8.0, 50 m*M* NaCl, and 1% NP-40) containing Roche complete protease inhibitor (Cat No. 11697498001, Roche). The protein samples were quantified with Bio-Rad protein assay dye (Cat No. 500-0006) and denatured with equal amount of 2× SDS loading buffer (0.125*M* Tris•Cl, pH 6.8, 4.6% SDS, 20% glycerol, 10% β-mercaptoethanol, and 0.1% bromophenol blue) at 100°C for 5 min. For immunodetection, 20 μg of total protein from each sample was loaded and separated in 7.5% SDS-PAGE gel and transferred to nitrocellulose membrane. Transgenic HSP70 was recognized with primary anti-myc antibody (Cat No. 11667203001, Roche) at a dilution of 1:5000 and appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Cat No. 170-6516, Bio-Rad) at 1:5000. α-Tubulin was used as internal loading control and was recognized by anti-α-Tubulin (ab7219, Abcam) at 1:5000. The ECL advance Western blotting detection kit (RPN2135, Amersham) was used for developing luminous fluorescence of specific bands. Results were visualized and analyzed with ChemiGenius bio-imaging system (Syngene, USA).

Locomotor Activity

Larval locomotion was video-recorded for subsequent analysis. The recording procedure was as follows. A large metal plate with 7 identical round chambers (diameter 6.0 cm, open bottom) was filled with 130 ml 1% non-nutritious agar. A few drops of black ink were added to the agar to generate contrast with the larvae and aid visualization. Eight to ten third-instar larvae were loaded in each chamber. For each Gal4/UAS combination, the control larvae (Gal4 or UAS-hsp70 alone) and the Gal4/UAS larvae were loaded in different chambers of the same plate. Before video-recording, larvae were placed on the agar surface for 5 min at room temperature, allowing them to adapt to the experimental conditions. Three minutes of crawling activity were then recorded with a Logitech QuickCam Pro 4000 digital camera (Logitech) with 640 × 480 resolution at 1 frame/sec. The plate was then placed on a water bath

so that the temperature of agar surface could be ramped to (40 ± 0.5)°C over 10 min. Recording started again immediately after putting the plate on the water bath and continued for 1 h. This recording time was determined by pretesting Canton-S (CS) larvae, most of which failed within about 25 min and the maximal time to failure was around 40 min. The recordings were repeated at least 4 times and around 30 larvae of each genotype were tracked.

To analyze locomotor ability, we compared the 3-min path length at 25°C and at 40°C. Crawling activity at 40°C was taken from the 1 h video. The 3-min time interval was taken after the 5 min adaptation period when temperature was sustained at 25 or 40°C. The time to movement failure (TMF) at 40°C was also measured. We defined "movement failure" as being when there was no locomotion and no visible minor movements such as backward moving, body rolling, or head swinging. Analysis of the 3-min path lengths and TMF was performed with Video Point 2.5 software (Lenox Softworks, MA). Three-minute path lengths were calculated by tracking the larval tail every 3 sec. TMF was measured during playback at 1 frame per 5 seconds (i.e. ±5 s).

Thermotaxis

This test was performed as described previously (Liu et al., 2003; Rosenzweig et al., 2005). An agar plate with gradient temperature ranging from 29 to 40°C was established. Twenty larvae were placed in the loading zone and a 5-min video-recording (0.1 frame/sec) was made immediately after loading. Three tests were performed for each larval genotype. Data were subsequently analyzed by replaying videos. The numbers of larvae on the hot side (N_h) and on the cool side (N_c) were counted after 2 min and after 5 min. The avoidance index (AI) was calculated as $AI = (N_c - N_h)/(N_c + N_h)$.

Immunostaining

Larvae were dissected to reveal the central nervous system and body wall muscle cells, and fixed in 4% paraformaldehyde. To stain the nervous system, preparations were incubated with Cy3 conjugated anti-HRP (Cat No. 123-165-021, Jackson ImmunoResearch) with 1:100 dilution at room temperature for 2 h. FITC-conjugated anti-myc (ab1393, Abcam) was used for immunostaining of transgenic HSP70 using a dilution of 1:100 at room temperature for 2 h. Fluorescence was visualized on an Axioplan 2 imaging universal microscope system (ZEISS, NY) and analyzed with Openlab 4.0.1 software (Improvision, MA). The number and size of type Ib boutons in muscle 6/7 at abdominal segment A2/3 were analyzed. To normalize the bouton size, we divided the bouton area by the area presented by muscle 6 in the same segment. For each genotype, 10 larvae were analyzed or the number is otherwise indicated.

Electrophysiology

Excitatory junction potentials (EJPs) were recorded at the neuromuscular junction (NMJ) of abdominal segment A2 or A3 in muscle 6. Larvae were dissected in calcium-free hemolymph-like saline (HL3) (Stewart et al., 1994). Recordings were made using glass microelectrodes (filled with 3*M* KAc; 25–60 MΩ) after replacing the calcium-free saline with HL3 containing 1 m*M* calcium. Saline perfusion allowed the preparation temperature to be increased and held at 36°C using a PTOC proportional temperature controller (Scientific Systems Design, Canada). Intracellular potentials were amplified using a Neuroprobe 1600 amplifier (A-M Systems, WA) and digitized with a Digidata 1322A (Axon Instruments, CA). Signals were acquired and analyzed with pClamp 9.0 software (Molecular Devices Corporation, CA).

EJP amplitudes were corrected for nonlinear summation as previously described (Feeney et al., 1998). Briefly, two different correction equations (Stevens, 1976; McLachlan and Martin, 1981) were employed and the average of two corrected EJP amplitudes was used for statistical comparison. The correction equation derived by McLachlan and Martin (1981) is

$$v' = v/(1 - f[v/E]) \quad (1)$$

where v' is the corrected amplitude, v is the measured amplitude. E is the driving force (difference between resting membrane potential and reversal potential, with the reversal potential estimated in this case to be 0 mV), and f is the membrane capacitance factor ($\Delta t/\tau$) (where Δt is the duration of transmitter action (30 ms) and τ is the membrane time constant (160 ms)).

The correction equation derived by Stevens (1976) is

$$v' = E(\ln[E/(E - v)]) \quad (2)$$

where v, v', and E are the same as in Eq. (1).

Heat Shock

Bottles containing third-instar larvae were placed in a water bath at 36°C for 1 h and subsequently removed to allow recovery at room temperature (25°C) for 1 h before collecting larvae or performing experiments.

Quantitative PCR

Total RNA from 10 larvae of each genotype was extracted with RNAqueous Kit (Cat No. 1912, Ambion). Containment DNA was removed with DNA free kit (Cat No. 1906, Ambion). The total RNA concentration was quantified and adjusted to 60 ng/μl. Seven microliter total RNA was then reverse transcribed to a final volume of 20 μl cDNA with SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Using 1 μl cDNA as template, quantitative PCR reactions (25 μl) were carried out with QuantiTect SYBR Green PCR Kit (Cat No. 204143, QiaGen) on a 7500 Fast Real-Time PCR System (AB Applied Biosystems). The *hsp70* and *hsp70-myc* cDNA were amplified. Serial dilutions of pINDY5-hsp70 plasmid

were used as standards to quantify *hsp70* and *hsp70-myc* according to the absolute quantification protocol (Applied Biosystems 7300/7500 Real Time PCR System). The primer design strategy was as follows: the forward primer was located in *hsp70* CDS and both *hsp70* and *hsp70-myc* shared this primer; the reverse primer for *hsp70* was also in *hsp70* CDS, while the reverse primer for *hsp70-myc* was located in *myc* tag region. Primer pairs were CAAGAACTCCGTCTTGGACAAGTG (forward) and TGCATCTTGGTCATGATAGGGG (reverse) for *hsp70*, CAAGAACTCCGTCTTGGACAAGTG (forward) and CGAGATCAACTTCTGTTCCATCG (reverse) for *hsp70-myc*. The amplification fragments were 135 bp (*hsp70*) and 280 bp (*hsp70-myc*). Samples were duplicated and each sample was loaded twice.

Statistics

Data were collected and analyzed using SigmaStat3.1 (Systat Software). Results were present as mean ± SEM. Appropriate statistical tests were applied as described in the text and significance was assumed at $p < 0.05$ (*) or $p < 0.01$ (**).

RESULTS

Motoneuronal HSP70 is Sufficient to Confer Thermoprotection at High Temperature

The UAS-hsp70 fly strains carried one copy of the *Drosophila hsp70*Ab gene with a c-myc tag sequence. The transgenic product, HSP70-myc, contains amino acid residues of HSP70 (642 amino acids), myc (10 amino acids), and a linker sequence (9 amino acids) [Fig. 1(A)]. The apparent molecular weight of HSP70-myc is 72 kDa. Transgenic HSP70 was clearly present in Gal4/UAS combination larvae, whereas no transgenic HSP70 was detectable in control larvae (UAS or Gal4 alone) [Fig. 1(B)].

We first examined the role of HSP70 on larval locomotor activity at room temperature (25°C) and at high temperature (40°C). Three Gal4 drivers were selected for directing HSP70 ubiquitously (da-Gal4), in motoneurons (D42-Gal4) or in muscles (24B-Gal4), and locomotor path lengths made during 3 min were compared. At room temperature, no difference of path length was observed between GAL4/UAS combination larvae and controls. There was also no significant difference between wild type (CS) and all the other transgenic animals [Fig. 1(C)]. However, at 40°C, the 3-min path length of da-Gal4/4.3 larvae was significantly longer than the path length traveled by the controls. The longer path length was also evident in da-Gal4/9.1 compared with controls [Fig. 1(C)]. Therefore, ubiquitously expressed HSP70 protected larval crawling ability at high temperature. Interestingly, at 40°C the path lengths of D42/4.3 and D42/9.1 (motoneuronal expression) were also longer than those of control larvae. However, directing HSP70 to muscles using 24B-Gal4 resulted in no differences in the path lengths of both 24B/4.3 and 24B/9.1 compared with their controls [Fig. 1(C)]. These data indicate that directing HSP70 to motoneurons was sufficient to protect larval crawling ability at high temperature, whereas directing HSP70 to muscles conferred no protection of crawling ability at high temperature.

To further address the role of HSP70 in protecting larval locomotor activity at high temperature, we video-recorded and analyzed larval crawling behavior at 40°C until movement failure. We found that in larvae with ubiquitous expression of HSP70 the TMF was significantly extended [Fig. 2(A,B)]. In larvae with HSP70 in motoneurons [Fig. 2(C,D)], but not in larvae with HSP70 in muscles [Fig. 2(E,F)], the TMF was also extended. These results confirm that ubiquitous HSP70 conferred protection of larval crawling behavior against high temperature (40°C), and that directed expression of HSP70 in motoneurons only was sufficient to confer the thermoprotection.

HSP70 has No Effect on Larval Thermotaxis

In a temperature preference assay, wild type *Drosophila* larvae exhibit strong thermotactic behavior (Rosenzweig et al., 2005). The larvae can rapidly sense high temperature and migrate down the thermal gradient to a cooler zone. A *Drosophila* homolog of a heat-activated transient receptor potential (TRP) family ion channel, *dTrpA1*, is essential for thermotaxis. HSP70 is not an ion channel protein. However, ubiquitous or motoneuronal HSP70 conferred thermoprotective locomotor behavior at high temperature. It is thus possible that HSP70 may also regulate larval thermotaxis. Here we examined this possibility. We directed HSP70 ubiquitously, in motoneurons or in muscles by crossing with the appropriate Gal4 drivers. Third-instar larvae were placed in the loading zone (the middle of an agar plate) and experienced a temperature gradient of 28.9–39.8°C [Fig. 3(A,B)]. Larvae initially wandered to either side but then quickly reoriented to the cool side. The number of larvae on each side was scored at 2 min and at 5 min. An AI was calculated as described in the Materials and Methods. There was no difference of AI between Gal4/UAS combination larvae and controls (UAS or Gal4 alone) at 2 min or 5 min, and there was no difference between CS and transgenic larvae [Fig. 3(C)]. Therefore, all the larvae were able to detect high temperature and perform successful avoidance behavior within 2 min, indicating that HSP70 had no effect on larval thermotaxis.

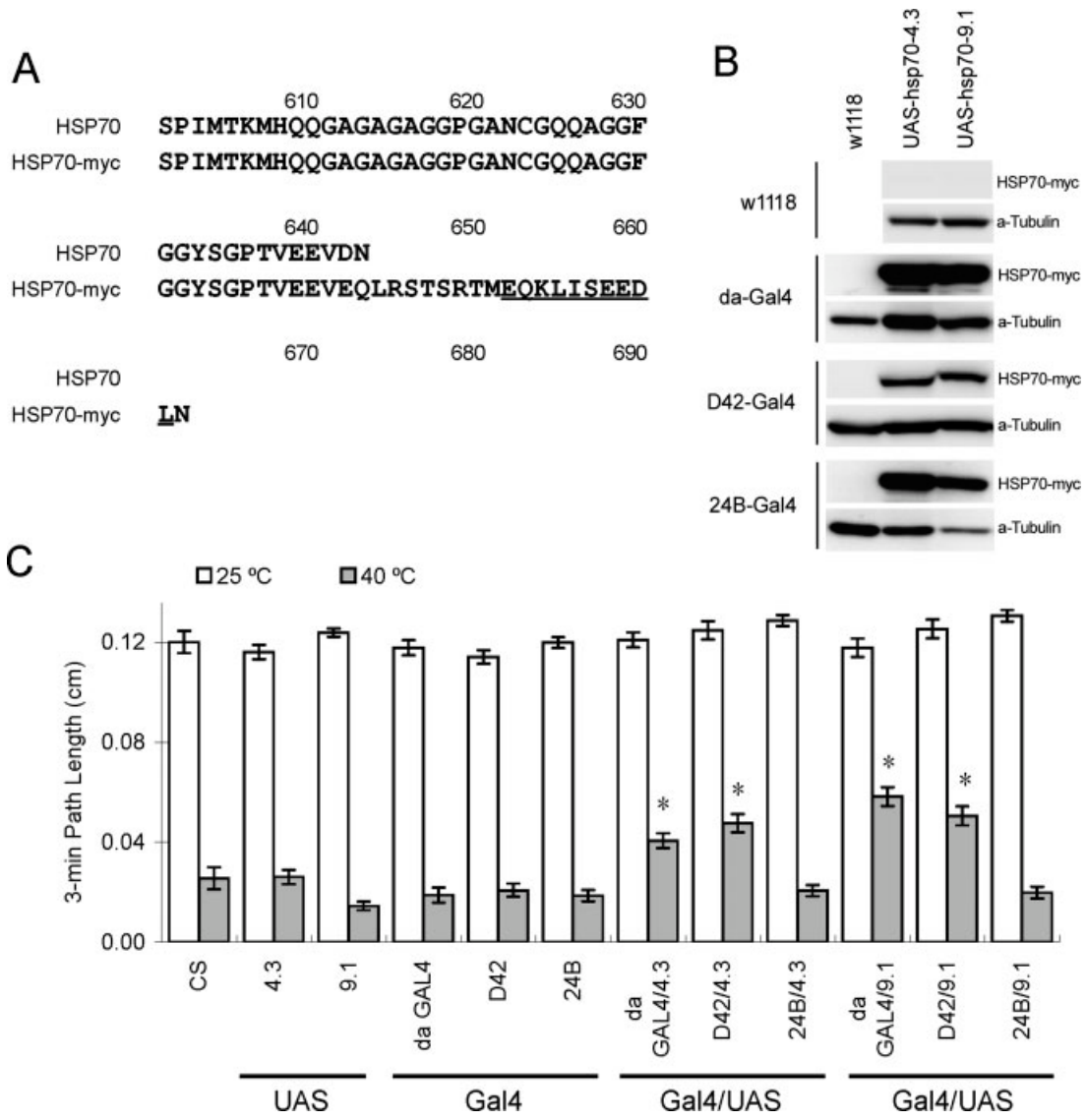

Figure 1 Directing HSP70 to motoneurons is sufficient to elicit thermoprotection at high temperature. (A) Carboxyl terminals of HSP70 (642 amino acids) and HSP70-myc (661 amino acids). HSP70-myc contains a Myc tag (underlined) and a linker sequence between HSP70 and Myc. The apparent molecular weight of HSP70-myc is 72 kDa. (B) Expression of transgenic HSP70 under the control of different tissue-specific GAL4 drivers in two UAS-hsp70 lines (4.3 and 9.1). The transgenic HSP70 was detected in whole larval protein extract with monoclonal anti-myc primary antibody (1:5000). α-Tubulin was used for a loading control and was detected by monoclonal anti-α-Tubulin (1:5000). (C) Three-minute path lengths of third-instar wandering stage larvae at room temperature (25°C) and at high temperature (40°C). Data were analyzed by two-way ANOVA and are presented as mean ± SEM. Asterisks (*) indicate significant differences from control values at the same temperature; $p < 0.05$.

Motoneurons are Critical for Originating HSP70-Mediated Thermoprotection

We observed that ubiquitously directing HSP70 extended the average TMF [Fig. 4(A,B)], and directing HSP70 to motoneurons [Fig. 4(E,F)] but not muscles [Fig. 4(C,D)] was sufficient to extend the average TMF, thus conferring thermoprotection of larval crawling behavior at high temperature. D42 driver is not purely motoneuron-specific at the third-instar larval stage (Parkes et al., 1998). Hence we used another predominantly motoneuron-specific driver OK6-Gal4 (Aberle et al., 2002) to direct HSP70 to motoneurons and examined locomotor

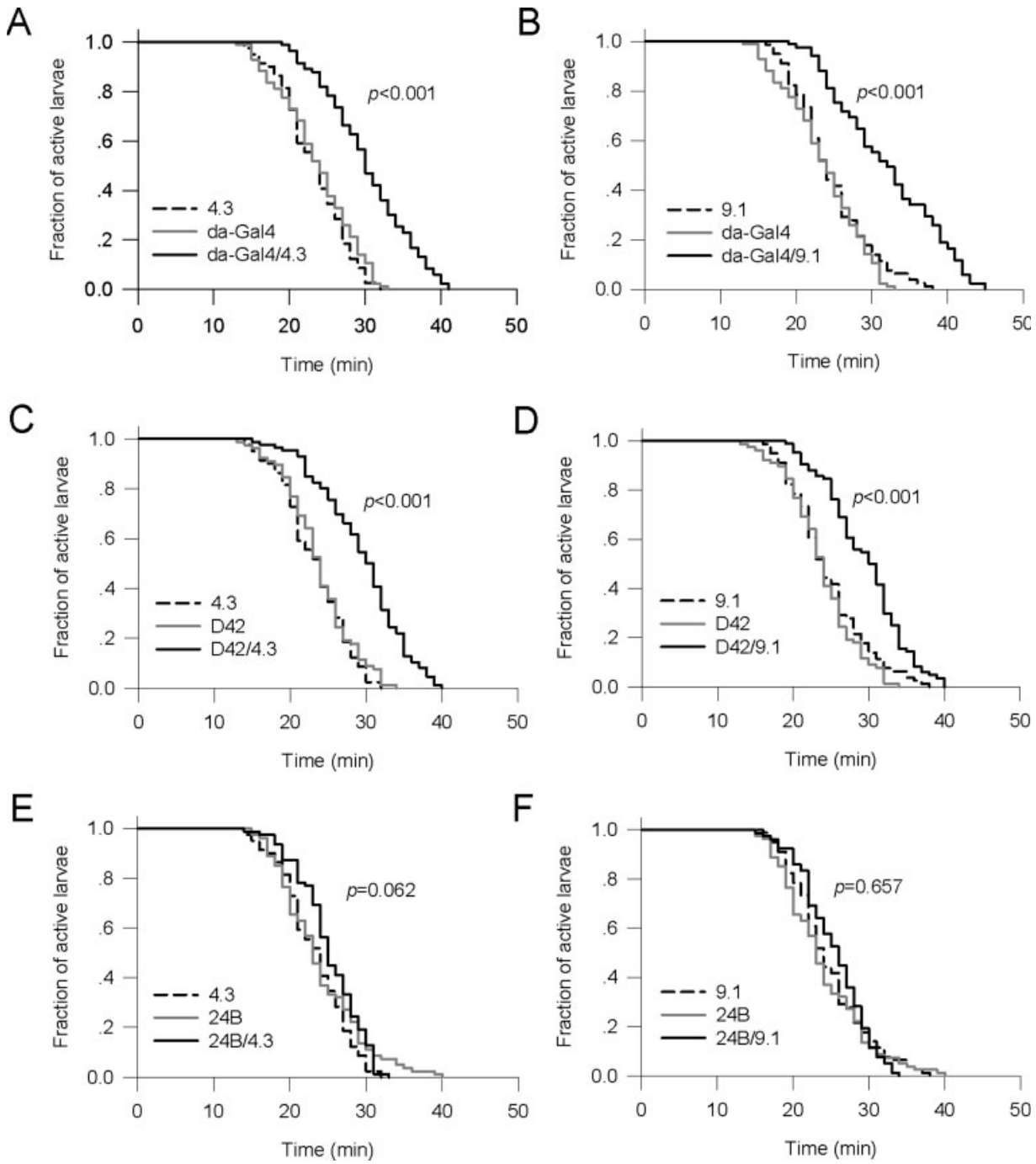

Figure 2 TMF at high temperature (40°C). The fraction of active larvae versus time was plotted using Kaplan–Meier survival analysis (Log-Rank). Two UAS-hsp70 lines (4.3 and 9.1) were crossed to da-Gal4 (ubiquitous; A, B), D42 (motoneuronal; C, D), and 24B (muscle; E, F). Each Gal4/UAS and the controls (Gal4 and UAS alone) were compared and *p* values are indicated. The data demonstrate that ubiquitous expression of HSP70 extended TMF in both 4.3 and 9.1 lines. Also, motoneuronal HSP70 (C, D) but not muscle HSP70 (E, F) was sufficient to extend TMF at high temperature.

behavior at room temperature and high temperature. Tissue-specific expression common to both D42-Gal4 and OK6-Gal4 was evident in motoneurons only. Relative HSP70 levels were a little lower in OK6-Gal4/UAS larvae (0.34 in OK6-Gal4/4.3, 0.32 in OK6-Gal4/9.1) than in D42-Gal4/UAS (0.43 in D42-

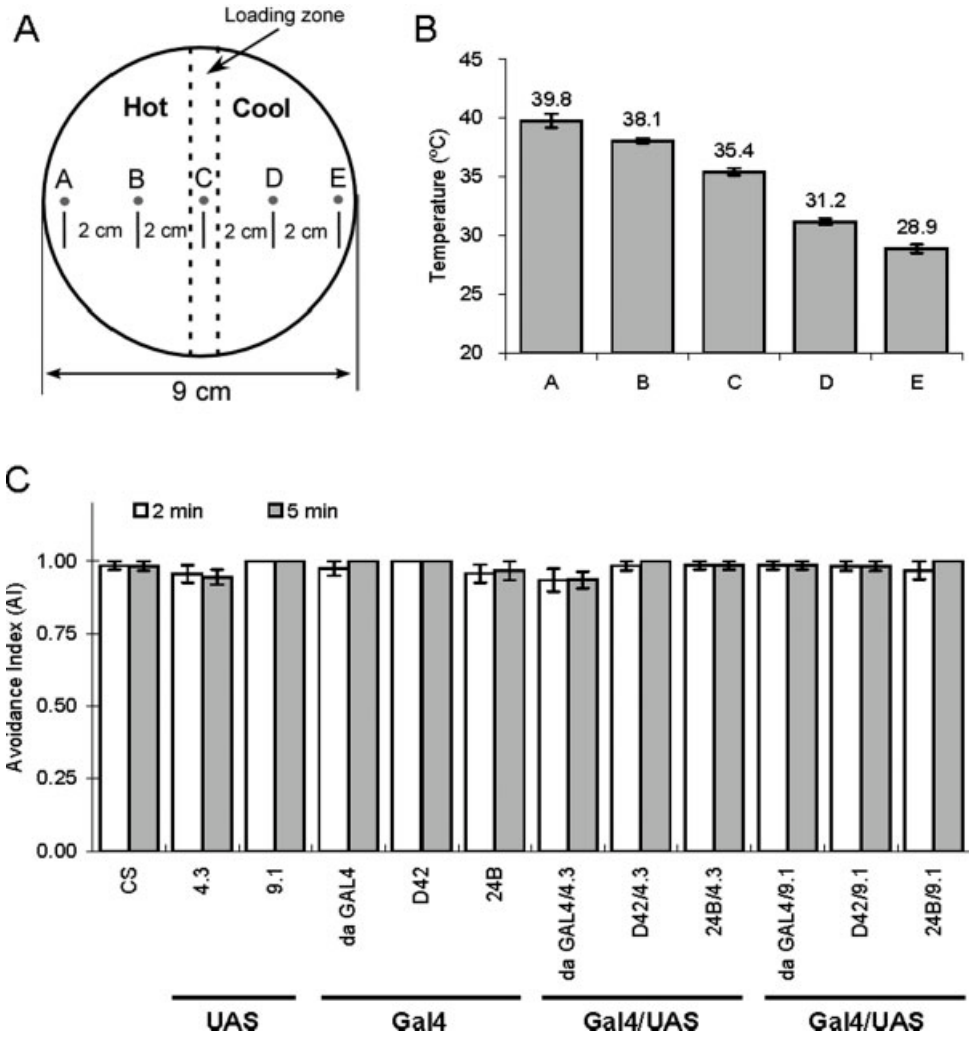

Figure 3 HSP70 has no effect on larval thermotaxis. (A) An agar plate (diameter 9 cm) with a thermal gradient was used to examine larval thermotactic behavior. (B) Temperatures measured at different positions demonstrate a thermal gradient from 39.8 to 28.9°C. (C) AI measured at 2 min or in 5 min was calculated as described in Materials and Methods. No significant difference was observed in Gal4/UAS and Gal4 or UAS alone, and no significant difference was observed between wild type (CS) and all the transgenic larvae. Thus, Gal4/UAS larvae demonstrated normal thermotactic behavior. Results are presented as mean ± SEM.

Gal4/4.3, 0.49 in D42-Gal4/9.1) [Fig. 4(E,G)]. The TMF was extended in both OK6-Gal4/4.3 ($p < 0.01$) and OK6-Gal4/9.1 ($p < 0.05$), which was consistent with the TMF extension in both D42-Gal4/4.3 ($p < 0.01$) and D42-Gal4/9.1 ($p < 0.01$) [Fig. 4(F,H)]. Therefore, with two motoneuron-specific drivers we found that motoneuronal HSP70 was sufficient to elicit thermoprotection at high temperature.

We then determined whether other neuronal subtypes could demonstrate the same role as motoneurons in eliciting HSP70-mediated thermoprotection. We examined the roles of peripheral sensory neurons, dopaminergic neurons, and serotonergic neurons in originating HSP70-mediated thermoprotection. To examine the role of all sensory neurons in originating thermoprotection, we directed HSP70 to the peripheral nervous system with P0163-Gal4 (Hummel et al., 2000). The results showed no TMF extension in spite of HSP70 expression in P0163-Gal4/4.3 or P0163-Gal4/9.1 larvae [Fig. 4(I,J)], indicating that sensory neurons alone had no effect in developing HSP70-mediated thermoprotection. The relationship between peripheral sensory neurons and locomotor pattern is intriguing. Sensory input is not necessary for the development of circuitry for larval crawling, but is required for this circuitry to generate normal patterns of larval locomotion

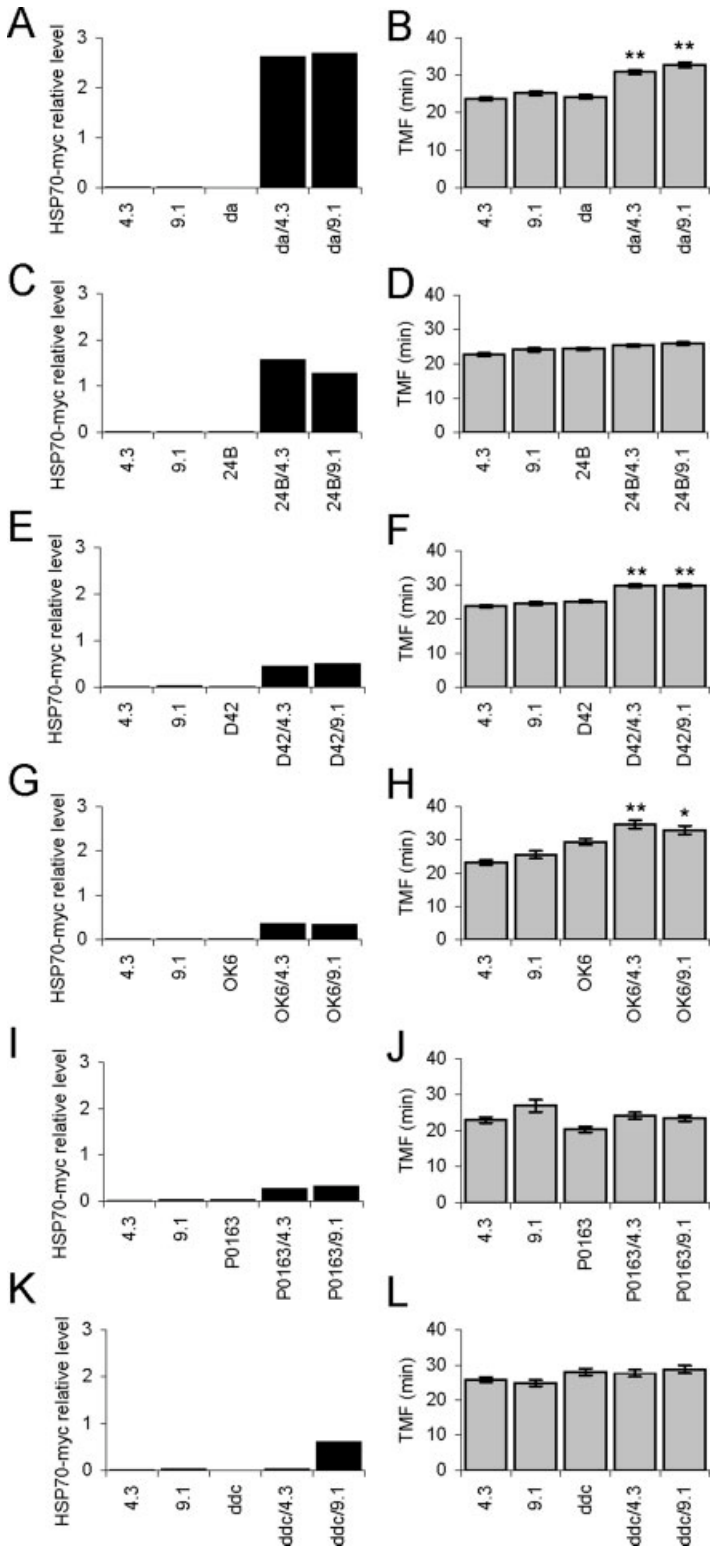

Figure 4 Motoneurons are critical for HSP70-mediated thermoprotection. Transgenic HSP70 was detected using the same experimental procedures as for Figure 1(B). TMF at 40°C was analyzed as described in Materials and Methods. Shown are the levels of transgenic HSP70 relative to the loading control (α-Tubulin) and the average TMF for two UAS-hsp70 lines (4.3 and 9.1) driven by da-Gal4 (A, B), 24B-Gal4 (C, D), D42-Gal4 (E, F), OK6-Gal4 (G, H), P0163-Gal4 (I, J), and ddc-Gal4 (K, L). Ubiquitously expressed HSP70 (da-Gal4) extended larval TMF significantly (B). Motoneuronal HSP70 was sufficient to extend TMF, and this was confirmed using two independent motoneuron-specific drivers, D42-Gal4 (F) and OK6-Gal4 (H). Driving HSP70 in peripheral sensory neurons (P0163-Gal4), and dopaminergic, and serotonergic neurons (ddc-Gal4) resulted in no TMF extension. TMF is presented as mean ± SEM. $^{**}p < 0.01$. $^{*}p < 0.05$.

(Suster and Bate, 2002). We observed that directing HSP70 to sensory neurons alone caused no thermoprotection of locomotor activity, indicating either that HSP70 did not protect sensory ability, or that sensory function was indeed protected but locomotor failure was due to the failure of central pattern generation or the motor output pathway at high temperature.

We also examined locomotor activity in larvae with HSP70 directed to dopaminergic and serotonergic neurons using ddc-Gal4 (Li et al., 2000). Expression of HSP70 in these neurons was detectable, but conferred no locomotor thermoprotection [Fig. 4(K,L)]. Hence dopaminergic and serotonergic neurons on their own showed no role in HSP70-mediated thermoprotection of locomotion. Dopaminergic and serotonergic neurons are interneurons in central nervous system (Li et al., 2000). These neurons are known to have a role in behavioral modulation, neuroendocrine activity, and development in *Drosophila* (Cooper and Neckameyer, 1999). Degeneration of dopaminergic neurons causes a common movement disorder named Parkinson's disease in mammals and human (Lang and Lozano, 1998). In *Drosophila*, directing a human HSP70 to dopaminergic neurons maintained the neuron number and prevented degeneration (Auluck et al., 2002; Bonini, 2002). Targeted expression of HSP70 to dopaminergic and serotonergic neurons had no thermoprotective effect on locomotor activity. This suggests that HSP70 in these neurons may prevent degeneration under certain circumstances but shows little role in maintaining any functional role of these neurons in locomotion. In addition, dopaminergic and serotonergic neurons are upstream to the motoneurons in the neuronal signaling pathway. The HSP70-mediated modulation in these neurons may not be enough to protect motoneuronal output at high temperatures. Thus dopaminergic and serotonergic neurons alone are insufficient in eliciting HSP70-mediated thermoprotection.

We conclude that motoneurons are the critical neuronal subtype in the nervous system for eliciting HSP70-mediated thermoprotection of locomotion, whereas peripheral sensory neurons, dopaminergic and serotonergic neurons alone are not sufficient in eliciting HSP70-mediated thermoprotective behavior.

Expression of HSP70 Causes Presynaptic Structural Plasticity

We found that motoneurons were critical for eliciting HSP70-mediated thermoprotection of locomotor activity, suggesting that their functional properties had been modified. Gal4 continuously activates UAS in Gal4/UAS larvae (Brand and Perrimon, 1993; McGuire et al., 2004) and thus HSP70 was constitutively expressed in motoneurons throughout embryonic and larval development. It has been shown that HSP70 is necessary only under stressful conditions and under normal conditions the basal HSP70 level is low because *hsp70* genes are transcriptionally inactive (Lindquist and Craig, 1988). Hence a high constitutive level of HSP70 might result in structural and functional changes of presynaptic boutons. Such changes are evident in a study demonstrating that the cell adhesion molecule Fasciclin II (Fas II) controls presynaptic structure plasticity. The *Fas II* mutation caused a significant increase of bouton number but with reduced quantal content in single boutons; the synaptic strength remained unchanged (Schuster et al., 1996). To characterize the effects of chronic HSP70 expression we examined changes in the morphology of motoneurons in Gal4/UAS larvae. Immunostaining demonstrated that HSP70-myc was present in motoneuronal somata, segmental nerves, and terminal boutons in D42-Gal4/4.3 larvae (see Fig. 5). There was no obviously preferential localization of HSP70-myc in motoneurons (see Fig. 5). We compared the number of boutons as well as the size of Type Ib boutons in muscle 6/7 at abdominal segment A2/3 [Fig. 6(A)]. In D42-Gal4/4.3 larvae the normalized number of Type Ib boutons ($19.1 \pm 2.0 \times 10^{-5}$, normalized to surface area of muscle 6 in the same abdominal segment) showed no difference as compared to controls ($11.9 \pm 1.3 \times 10^{-5}$ in 4.3 and $17.3 \pm 1.1 \times 10^{-5}$ in D42) [Fig. 6(B)], but the average bouton size in D42-Gal4/4.3 ($18.8 \pm 1.5 \times 10^{-5}$) was bigger than that in controls ($12.7 \pm 1.4 \times 10^{-5}$ in 4.3 and $11.6 \pm 1.2 \times 10^{-5}$ in D42) ($p < 0.01$, one-way ANOVA) [Fig. 6(C)]. In particular, the number of large boutons ($30–50 \times 10^{-5}$) increased in D42-Gal4/4.3 larvae compared with controls [Fig. 6(D)]. We also calculated the cumulative bouton area in each genotype. The cumulative bouton area in D42-Gal4/4.3 (938.0×10^{-5}) was around twofold higher than that in 4.3 (413.5×10^{-5}) or D42 (475.5×10^{-5}) [Fig. 6(E)].

To determine whether the increased bouton size and cumulative bouton area are specifically caused by Hsp70, we also examined the motor terminal morphology in D42-Gal4/UAS-GFP larvae ($n = 5$). We found that both normalized bouton number and bouton size remained unchanged in D42-Gal4/UAS-GFP larvae compared to D42 [Fig. 6(B,C)]. GFP protein also showed no effects on different-sized boutons [Fig. 6(D)] and cumulative bouton area [Fig. 6(E)]. Thus, the increased bouton size and cumulative bouton area are more specifically caused by Hsp70. Taken together, in D42-Gal4/4.3 larvae chronic expression of transgenic HSP70 caused more large boutons with increased cumulative bouton area in motoneuronal terminals.

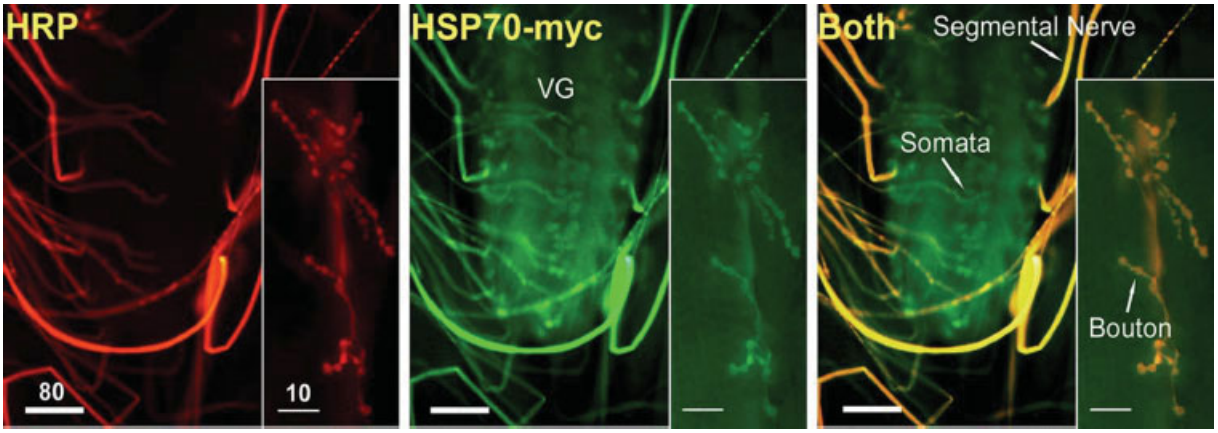

Figure 5 Double immunostaining of D42/4.3 larval central nervous system. The larval fillet dissection was stained with Cy3-anti-HRP (1:100) and FITC-anti-myc (1:100) at room temperature for 2 h. HRP was strongly stained in segmental nerves and terminal boutons. HSP70-myc was clearly shown in somata, segmental nerves, and terminal boutons. Scale bar unit: μm.

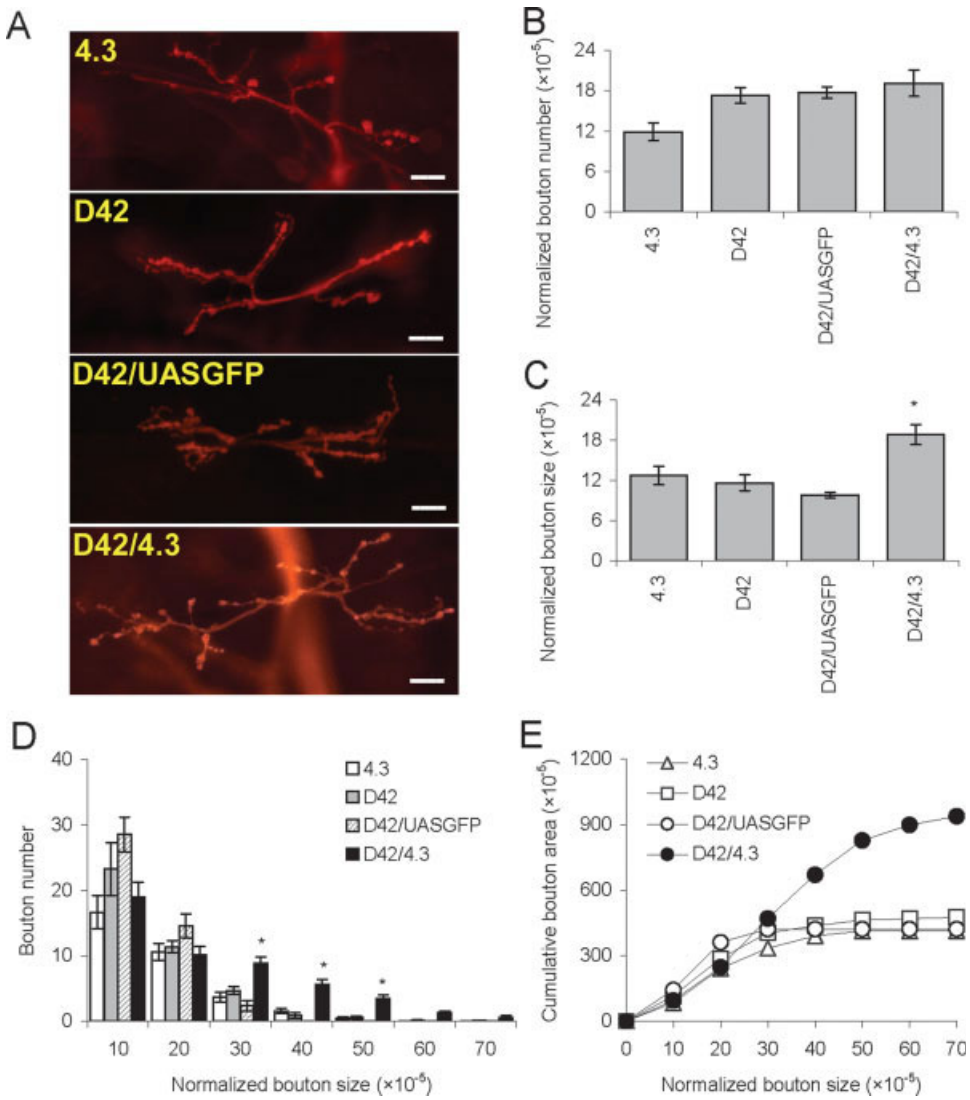

Figure 6 (see legend on following page)

To determine whether acute heat shock has similar effects on motoneuronal structure as chronic HSP70 expression, we made the same measurements in wild type w1118 nonheat shock (NHS, $n = 4$) and heat shock (HS, $n = 4$) larvae. There were no significant differences in bouton number (NHS, 46.0 $\pm$ 3.3; HS, 45.4 $\pm$ 1.8), average bouton size relative to area of the abdominal muscle fiber 6 (NHS, 10.1 $\pm$ 0.7 $\times$ 10^{-5}; HS, 10.6 $\pm$ 0.8 $\times$ 10^{-5}) or relative cumulative bouton area (NHS, 455 $\times$ 10^{-5}; HS, 477 $\times$ 10^{-5}).

HSP70 Protects Quantal Content of Neurotransmission at High Temperature

To test the possibility that HSP70 causes functional plasticity of presynaptic boutons, for example whether the quantal content was reduced or remained unchanged, we recorded evoked EJPs and miniature excitatory junction potentials (mEJPs) at the NMJ in abdominal muscle 6 of segment A2 or A3. The corrected EJP amplitude in D42/4.3 showed no increase at 25°C but a significant increase at 36°C (17.0 $\pm$ 1.4 mV, $n = 8$ in D42/4.3, compared with 10.9 $\pm$ 1.5 mV, $n = 7$ in 4.3, or 8.7 $\pm$ 1.9 mV, $n = 9$ in D42) ($p < 0.05$, one-way ANOVA) [Fig. 7(A,E)]. In each genotype, mEJP amplitudes reduced as temperature increased from 25 to 36°C. However, there was no significant difference of mEJP amplitude in D42/4.3 observed at 25°C or at 36°C [Fig. 7(B)]. There was also no statistical difference of the frequency of mEJP in D42/4.3 at either 25 or 36°C [Fig. 7(C)]. The average quantal content (corrected EJP amplitude/mEJP amplitude) at room temperature remained unchanged in D42/4.3, but was significantly higher at high temperature (32.9 $\pm$ 2.3 in D42/4.3, $n = 8$) than in controls (18.5 $\pm$ 1.8 in 4.3, $n = 7$; 19.4 $\pm$ 2.4 in D42, $n = 9$) ($p < 0.05$, one-way ANOVA) [Fig. 7(D)]. The lack of an increase in EJP amplitude at room temperature in D42/4.3 indicates that evoked transmitter release at active sites may actually be reduced because both average bouton size and cumulative bouton area significantly increased [Fig. 6(D,E)]. However, the significantly higher EJP amplitude at high temperature indicates clearly that EJP amplitude was protected from hyperthermia. Moreover, the higher quantal content at 36°C but not at 25°C demonstrates that HSP70 protected the release of quanta only at high temperature. Thus, we conclude that HSP70 protected synaptic transmission at high temperatures by preventing a loss of quantal content.

HSP70 Mimicks Heat Shock to Elicit Thermoprotection but There is No Cumulative Effect

It is well established that a brief heat shock induces synthesis of HSPs, predominantly HSP70, and elicits acquired thermotolerance in organisms (Lindquist, 1992). Here we found that thermoprotection of locomotion can be elicited by targeting a single HSP to motoneurons only. This HSP70-mediated thermoprotection might be a useful model for understanding the molecular mechanism of thermoprotection because HSP70-mediated thermoprotection avoids the pleiotropic effects caused by heat shock. Since HSP70 is involved in both HSP70-mediated thermoprotection and heat shock-induced thermoprotection, we were interested in determining whether (1) HSP70-mediated thermoprotection originating from motoneurons demonstrates the same level of protection as heat shock-induced thermoprotection in larval locomotor activity, (2) the effects of HSP70-mediated thermoprotection and heat shock-induced thermoprotection could be cumulative, and (3) transgenic HSP70 can down-regulate induction of endogenous HSP70.

We directed HSP70 to motoneurons by combining D42-Gal4 and 4.3, and recorded the TMFs at 40°C in larvae with or without prior heat shock conditioning (36°C, 1 h; 25°C, 1h). In larvae with 4.3 or D42

Figure 6 Motoneuronal HSP70 causes structural plasticity of axonal terminals. (A) Shown are typical terminal boutons stained with Cy3-anti-HRP (1:100) on muscle 6 and 7 at abdominal segment 2/3 in four genotypes. Scale bar is 40 μm. The normalized number of boutons (B), bouton size (C, D), and cumulative bouton area of Type Ib boutons (E) were compared. The bouton size was normalized to the area of muscle 6 in the same segment. The number of Type Ib boutons in D42-Gal4/4.3 larvae showed no significant difference from controls (B). However the average bouton size increased in D42-Gal4/4.3 (C). Specifically, the numbers of large boutons (30–50 $\times$ 10^{-5}) were significantly increased (D). The cumulative bouton area was significantly higher in D42-Gal4/4.3 than that in 4.3 or D42-Gal4 (E). Targeting GFP to motoneurons caused no significant increase of normalized bouton number (B) and size (C, D). GFP also caused no effect on cumulative bouton area (E) as compared to D42-Gal4. Plotted as mean $\pm$ SEM.

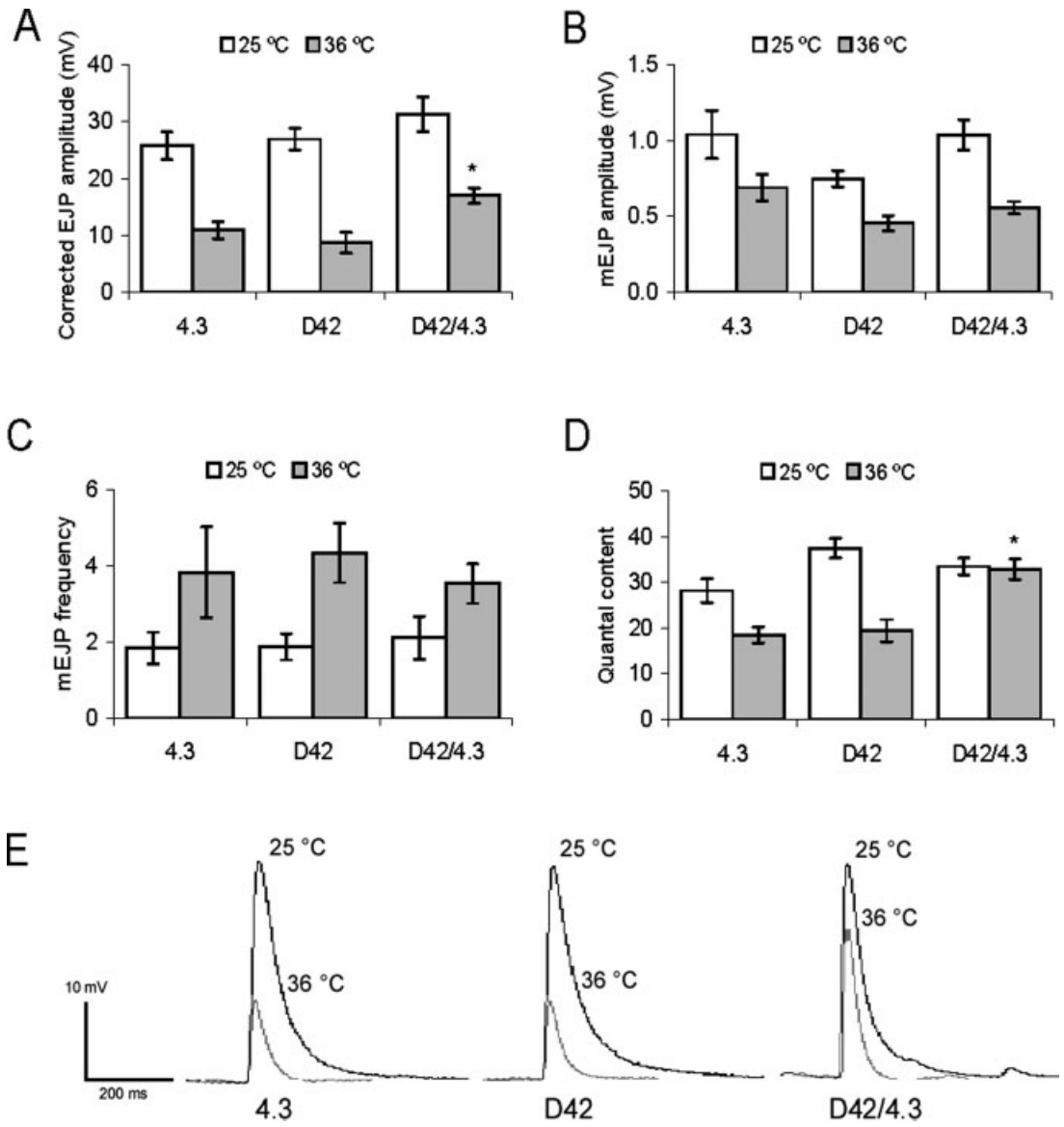

Figure 7 HSP70 protects quantal content of neurotransmission at high temperature (36°C). (A) Average EJP amplitude of three genotypes of larvae. EJPs were corrected for nonlinear summation as described in Material and Methods. The EJP amplitudes in D42/4.3 were not significantly increased at 25°C but significantly increased at 36°C ($p < 0.05$, one-way ANOVA). Spontaneous miniature EJP (mEJP) amplitude (B) and frequency (C) at room temperature (25°C) and high temperature (36°C) were analyzed. There was no significant change of mEJP amplitude or frequency in D42/4.3 in either 25 or 36°C. (D) Average quantal content in D42/4.3 (32.9 ± 2.3, $n = 8$) was higher than that in controls (18.5 ± 1.8, $n = 7$ in 4.3; 19.4 ± 2.4, $n = 9$ in D42) ($p < 0.05$, one-way ANOVA) at 36°C but not at 25°C. Quantal content was calculated as corrected EJP/mEJP from same preparation. (E) Typical EJP recordings from three groups of preparations. Plotted as mean ± SEM.

alone, prior heat shock resulted in significant increases of TMF ($p < 0.01$, two-way ANOVA), indicating that heat shock-induced thermoprotection occurred as normal in these larvae [Fig. 8(A)]. In D42/4.3 larvae, the TMF without prior heat shock was comparable with heat-shocked D42 larvae (29.53 ± 1.09 vs. 33.74 ± 1.38 min, $p = 0.054$) or heat-shocked 4.3 (29.53 ± 1.09 vs. 31.35 ± 1.13 min, $p = 0.29$) [Fig. 8(A)]. The results indicate that HSP70-mediated thermoprotection mimicks this measure of

heat shock-induced thermoprotection under these heat shock conditions (36°C, 1 h; 25°C, 1 h).

Then we examined whether there was an additive effect of motoneuron-originated HSP70-mediated thermoprotection and heat shock-induced thermoprotection when applying heat shock to D42/4.3 larvae. We found that TMF in D42/4.3 larvae (33.2 ± 0.8 min) after heat shock increased compared with TMF in nonheat shock (29.5 ± 1.1 min) ($p < 0.05$, two-way ANOVA). The heat shock-induced TMF was comparable in all three groups ($p = 0.43$, two-way ANOVA). These results demonstrate that heat shock elicited higher thermoprotection than motoneuronal HSP70 did, but no additive effect was observed in the heat-shocked D42/4.3 larvae. This indicates that HSP70-mediated thermoprotection may converge with heat shock-induced thermoprotection, and that HSP70 alone was not as strong as heat shock to induce thermoprotection. These results suggest that heat shock might induce HSP70 expression in tissues other than motoneurons and elicit a further thermoprotective effect. We tested this possibility by driving HSP70 ubiquitously. We found that ubiquitous expression of HSP70 extended TMF significantly in da-Gal4/4.3 (30.3 ± 0.7 min) compared with 4.3 (23.6 ± 0.5 min) or da-Gal4 (24.0 ± 0.1 min) ($p < 0.01$, two-way ANOVA). The HSP70-mediated TMF extension was comparable with heat shock-induced TMF extension with no statistical difference [Fig. 8(B)]. These data demonstrate that driving HSP70 ubiquitously can elicit the same thermoprotection as a heat shock pretreatment (36°C, 1 h; 25°C, 1 h). Again, there was no cumulative thermoprotection observed in the larvae with heat shock and ubiquitous expression of HSP70.

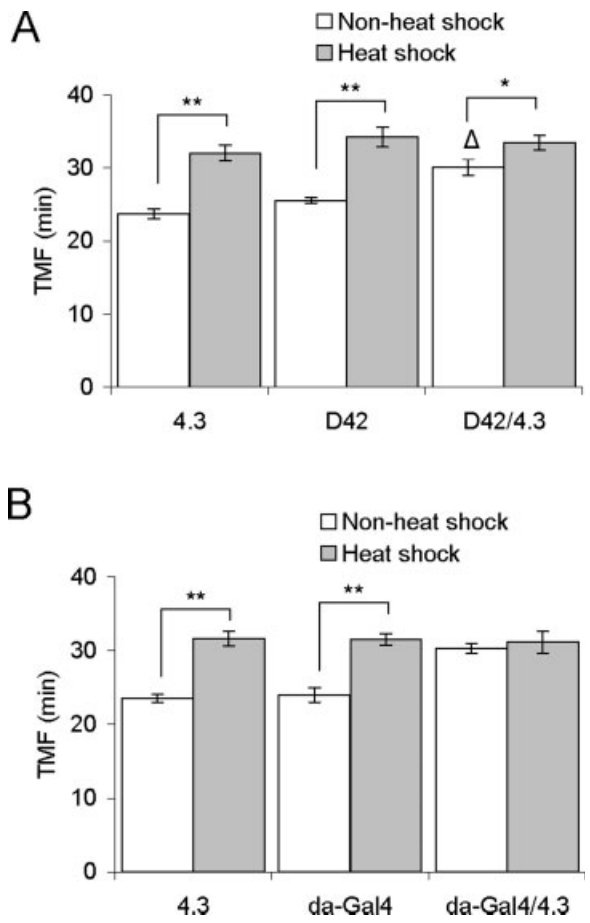

Figure 8 HSP70-mediated thermoprotection mimicks heat shock-induced thermoprotection but there is no additive effect. TMF at 40°C was analyzed under nonheat shock or heat shock conditions (36°C 1h, 25°C 1h) in two Gal4/UAS larvae and their controls. Without heat shock the TMF in D42/4.3 (30.1 ± 1.1 min) was longer than that in controls (23.7 ± 0.7 min in 4.3; 25.5 ± 0.4 min in D42) (Δ, $p < 0.01$, two-way ANOVA). Heat shock caused extended TMF in 4.3 (32.0 ± 1.1 min) and D42 (34.2 ± 1.4 min), and in D42/4.3 (33.4 ± 1.0 min) as well. After heat shock there was no significant difference of TMFs within three groups. The results show that heat shock induced further thermoprotection in D42/4.3 larvae, although TMF in D42/4.3 was already higher than in controls without heat shock. (B) Ubiquitous expression of HSP70 extended TMF without prior heat shock. Heat shock induced no further TMF extension in da-Gal4/4.3. Plotted as mean ± SEM.

Transgenic HSP70 Activates Transcription of Endogenous *hsp70*

One explanation for the previous results is that transgenic HSP70 down-regulates the induction of endogenous HSP70 in motoneurons, resulting in compensated but not additive thermoprotection. However, direct comparison of transgenic and endogenous HSP70 in motoneurons is difficult, unless motoneurons can be isolated from larva. Since heat shock induces the synthesis of HSP70 in all larval tissue, we compared endogenous and transgenic *hsp70* mRNA levels in da-Gal4/4.3 larvae, in which the transgenic HSP70 was ubiquitously targeted.

A two-step quantitative PCR was performed to detect transgenic and endogenous *hsp70* mRNA. Results demonstrated that the transgenic *hsp70* (*hsp70-myc*) mRNA level was very high in da-Gal4/4.3 larvae (91×10^5 copies/μl), compared with controls (0.05×10^5 copies/μl) in 4.3 and at an undetectable level in da-Gal4. After heat shock, there was no increase of *hsp70-myc* mRNA in da Gal4/4.3 larvae [Fig. 9(A,B)]. The *hsp70* mRNA level in da-Gal4/4.3 larvae (15×10^5 copies/μl) was also very high as compared to 4.3 (0.14×10^5 copies/μl) and da-Gal4 (0.06×10^5 copies/μl)

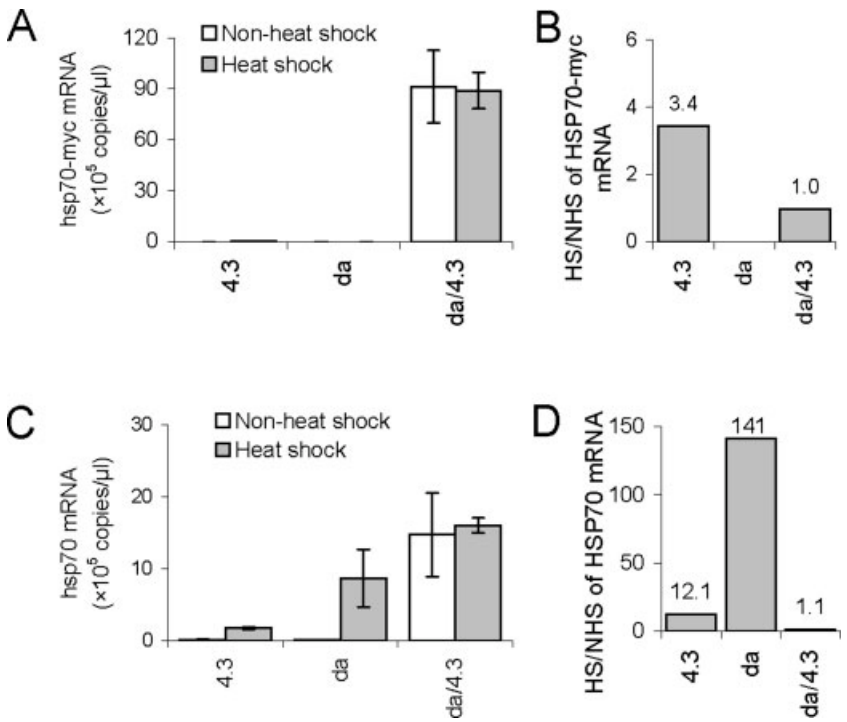

Figure 9 Transgenic HSP70 activates transcription of endogenous *hsp70*. Transgenic and endogenous *hsp70* mRNA was analyzed by quantitative PCR as described in Materials and Methods. (A) *hsp70-myc* mRNA accumulated to very high level (91×10^5 copies/μl). (B) After heat shock there was no increase (1.0-fold). (C) The *hsp70* mRNA level without heat shock was low in 4.3 (0.14×10^5 copies/μl) and da-Gal4 (0.06×10^5 copies/μl) but very high in da-Gal4/4.3 (15×10^5 copies/μl). (D) With heat shock *hsp70* mRNA showed almost no increase (1.1-fold), comparing to 12.1-fold increase in 4.3 and 141-fold increased in da-Gal4. The results demonstrate that transgenic HSP70 activated transcription of endogenous *hsp70* under nonheat shock conditions. Plotted as mean ± SEM.

without heat shock. After heat shock, in da-Gal4/4.3 larvae there was almost no increase (1.1-fold) of *hsp70* mRNA. In controls heat shock induced a 12.1-fold increase of *hsp70* mRNA in 4.3 larvae and a 141-fold increase in da-Gal4 [Fig. 9(C,D)]. The extremely high increase of *hsp70* mRNA after heat shock in da-Gal4 larvae may result from combined stresses of both heat shock and persistent, strong, and ubiquitous expression of Gal4 transcription factor. These results indicate that transgenic HSP70 almost fully activated endogenous *hsp70* genes without heat shock. Both *hsp70-myc* mRNA and *hsp70* mRNA showed almost no increase after heat shock.

Another reasonable explanation for the noncumulative thermoprotection in heat shocked da-Gal4/4.3 larvae is that the HSP70 level was saturated and more than the larvae need to elicit thermoprotection. Too much HSP70 has been reported to cause harmful effects on larval thermotolerance (Krebs and Feder, 1998). We found that, without heat shock, the combination of ubiquitously transgenic HSP70 and up-regulated endogenous HSP70 was sufficient to elicit thermoprotection to the same level as that induced by a prior heat shock (36°C, 1 h; 25°C, 1 h). We infer that in motoneurons, transgenic HSP70 may also up-regulate transcription of endogenous *hsp70* and, without prior heat shock, both transgenic and endogenous HSP70 elicit thermoprotection of larval locomotor activity.

DISCUSSION

There are 6 *hsp70* genes in the *Drosophila* genome (Gong and Golic, 2004). They are all inactive at room temperature and can be activated by heat shock (Lindquist and Craig, 1988; Bettencourt and Feder, 2002). We cloned one extra copy of *hsp70* which could be controlled using the GAL4/UAS system enabling us to examine the role of HSP70 in specific tissues without heat shock pretreatment. We found that targeting HSP70 to motoneurons alone protects lar-

vae from high temperature stress by enhancing locomotor activity. This thermal protection mimics heat shock-induced thermotolerance, although it may not be as strong as heat shock-induced thermotolerance, which may be due to the activation of all the endogenous *hsp70* genes and other *hsp* genes by heat shock. We also found that synaptic transmission at the larval NMJ was preserved as temperature increased in larvae with motoneuronal expression of HSP70. The apparently anomalous finding that locomotor ability was not protected but was impaired at high temperatures or after HS in transgenic flies with 12 extra copies of *hsp70* genes may result from excessive and ubiquitous HSP70 expression after heat shock (Klose et al., 2005). In a study using the same transgenic flies it was evident that abnormally high concentrations of HSP70 can decrease growth, development, and survival to adulthood (Krebs and Feder, 1997). Here we show that the levels of ubiquitous, motoneuronal, or muscular HSP70 expression produced using our UAS-hsp70 lines were not sufficient to impair normal thermotactic behavior. Thus we believe that the fly lines described here will be more effective for determining the normal mechanisms by which HSP70 protects neural function.

Targeting HSP70 to muscle cells alone failed to protect locomotor ability at 40°C. One explanation is that muscle cells are less vulnerable to hyperthermia, i.e., that motoneurons represent the weakest link in the motor pathway from central pattern generator to behavior. Alternatively, locomotor performance may be less vulnerable to impaired contractility of muscle cells than to a disturbance of neuronal output from motoneurons during hyperthermia. In addition, heat shock of different cell types may trigger different mechanisms leading to HSP70-mediated thermoprotection. Cell types that lose the ability for turnover, like postmitotic motoneurons, are likely to be more sensitive to the persistent effects of constitutively expressed HSP70. We also observed that motoneurons were the critical neuronal subtype in the nervous system for eliciting HSP70-mediated thermoprotection of locomotion. Tissue-specific HSP70 expression may have protected cellular function in peripheral sensory neurons, dopaminergic neurons, and serotonergic neurons but, if so, for each neuronal subtype this was not sufficient for eliciting HSP70-mediated locomotor thermoprotection. These findings indicate that the motor output pathway is a primary functional target for behavioral thermoprotection mediated by HSP70.

Since transgenic HSP70 is constitutively activated in motoneurons using the Gal4/UAS system (Brand and Perrimon, 1993; McGuire et al., 2004), it is perhaps not surprising that changes that require time to become evident, like the structural plasticity of axonal terminals, were observed. The morphological changes at motoneuronal terminals indicate that chronic expression of HSP70, arguably mimicking persistent thermal stress, restructured NMJs in a fashion that can be interpreted as being adaptive for high temperature. At room temperature the average bouton size and cumulative bouton area increased, whereas the total evoked transmitter release (EJP amplitude) was not significantly increased, thus the ability of transmitter release actually reduced. Therefore, at room temperature HSP70 compromised presynaptic function but at high temperatures this effect was compensated by structural plasticity (increased bouton size and cumulative bouton area) and functional preservation (greater EJP amplitude and quantal content). In this fashion HSP70 increased the operational scope for transmitter release to withstand high temperatures. Furthermore, quantal size remained unchanged among three groups of larvae at either room temperature or high temperature. Heat shock itself has been shown not to affect synaptic vesicle size as measured by examination of electron micrographs (Karunanithi et al., 1999). A plausible explanation for the HSP70-mediated thermoprotection of locomotion we observed is that HSP70 facilitates structural plasticity of motoneuronal terminals, with a concomitant increase in transmitter release at elevated temperatures, thus preserving the capability for motor output.

Morphological change is a long-term consequence of constitutively expressed HSP70 in motoneurons. This may involve the continuous and careful remodeling of cellular protein homeostasis. We demonstrate that transgenic HSP70 activated the transcription of endogenous *hsp70* genes under nonheat shock conditions. In other words, constitutively expressed HSP70 may function as a persistent cellular stressor to induce the motoneuronal stress response, in which the characteristic molecular change is the induction of endogenous HSP70. This, in turn, regulates the signal pathways leading to NMJ development and growth, restructuring motoneuronal terminals in the absence of thermal stress but enabling a presynaptic strengthening of motor output at high temperatures. In a similar fashion it has been observed that a higher rearing temperature (29°C) than normal increases bouton outgrowth, and that this is associated with elevated locomotor activity (Sigrist et al., 2003). Thus, structural plasticity seems to be a result of chronic heat stress or persistent expression of HSP70. After an acute heat shock (36°C, 1 h; 25°C, 1 h), we observed no significant increase of bouton number, average bouton size, and cumulative bouton area in w1118 strain. These results indicate that chronic

and acute heat stress may activate different mechanisms to modify motoneuronal terminal structure or physiology but with a similar functional thermoprotection. Such a two step strategy whereby long-term changes with structural modification follow short-term physiological changes is common in other models of synaptic plasticity (Hawkins et al., 2006).

A major question concerns the mechanism(s) by which HSP70 exerts its effects on synaptic morphology and physiology. The most important role of HSP70 is as a molecular chaperone to facilitate protein folding, assembly, and membrane translocation, and to degrade denatured, damaged, or aggregated proteins (Lindquist and Craig, 1988; Morimoto, 1993). The finding of more large boutons in HSP70 expression larvae might be explained by such chaperone functions of HSP70 acting to regulate the organization of motor system, particularly the axonal terminal outgrowth of motoneurons, by stabilizing cytoskeletal structures and protein complexes (Liang and MacRae, 1997). Conceivably chronic HSP70 prevents the pruning of some motoneuronal branches that would normally occur during development.

It is an interesting finding that transgenic HSP70 activates transcription of endogenous *hsp70*. Most probably, the cells recognize transgenic Hsp70 as an unwanted or ectopic protein and trigger cellular stress response and induce the expression of endogenous Hsp70, which may function more effectively to degrade or sequester transgenic Hsp70-myc. A fruitful strategy to address the relative roles of transgenic versus endogenous HSP70 would be to transgenically upregulate HSP70 in an endogenous HSP70 null background (Gong and Golic, 2004).

The molecular targets with which HSP70 interacts remain to be clarified. It is quite likely that, as a chaperone, HSP70 stabilizes several protein complexes essential for proper motoneuronal function, i.e. has more than one molecular target. A strong possibility is that HSP70 stabilizes the protein machinery controlling the synaptic vesicle exocytosis/endocytosis cycle by supplementing the activities of the HSP70 cognate, HSC70. The cognate protein participates in a trimeric protein complex, with cysteine string protein (CSP) and small glutamine-rich tetratricopeptide repeat protein (SGT), that acts as a synaptic vesicle chaperone machine (Tobaben et al., 2001). Genetic manipulations of CSP and HSC70 have profound effects on the thermosensitivity of synaptic transmission (Bronk et al., 2001; Bronk et al., 2005). In addition, HSP70 may be required to restore the function of the mutant dynamin (a protein essential for vesicle endocytosis) in temperature-sensitive *shibire* mutant. In flies lacking all copies of the *hsp70* genes, the recovery from paralysis after a return from a nonpermissive temperature is slowed and incomplete (Gong and Golic, 2006). Another possibility is that HSP70 is involved in stabilizing intracellular calcium homeostasis in motoneurons and that this is reflected in presynaptic thermoprotection. HSP70 binds to the sarco-endoplasmic reticulum Ca^{2+} ATP-ase (SERCA) of skeletal muscle and prevents thermal deactivation (Tupling et al., 2004), and HSP70 improves ischemia-impaired Ca^{2+} homeostasis in rat ventricular myocytes (Liu et al., 2006). Both of these studies indicate a role for HSP70 in stabilizing calcium dynamics. Finally, our own preliminary experiments demonstrate that prior heat shock can mitigate the disruptive effects of hyperthermia on Ca^{2+} homeostasis in presynaptic boutons of *Drosophila* (Klose et al., 2004). These potential roles for HSP70 are not mutually exclusive but further experiments are required to clarify exactly how HSP70 orchestrates its protective effects in motoneurons.

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