Synaptic Thermoprotection in a Desert-Dwelling *Drosophila* Species

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ABSTRACT: Synaptic transmission is a critical mechanism for transferring information from the nervous system to the body. Environmental stress, such as extreme temperature, can disrupt synaptic transmission and result in death. Previous work on larval *Drosophila* has shown that prior heat-shock exposure protects synaptic transmission against failure during subsequent thermal stress. This induced thermoprotection has been ascribed to an up-regulation of the inducible heat-shock protein, Hsp70. However, the mechanisms mediating *natural* thermoprotection in the wild are unknown. We compared synaptic thermosensitivity between *D. melanogaster* and a desert species, *D. arizonae*. Synaptic thermosensitivity and the functional limits of the related locomotor behavior differed significantly between closely related, albeit ecologically distinct species. Locomotory behavior of wandering third instar *D. arizonae* larvae was less thermosensitive and the upper temperature limit of locomotory function exceeded that of *D. melanogaster* by 6°C. Behavioral results corresponded with significantly lower synaptic thermosensitivity at the neuromuscular junction in *D. arizonae*. Prior heat-shock protected only *D. melanogaster* by increasing relative excitatory junctional potential (EJP) duration, the time required for EJP failure at 40°C, and the incidence of EJP recovery following heat-induced failure. Hsp70 induction profiles following heat-shock demonstrate up-regulation of inducible Hsp70 in *D. melanogaster* but not in *D. arizonae*. However, expression of Hsp70 under control conditions is greater in *D. arizonae*. These results suggest that the mechanisms of natural thermoprotection involve an increase in baseline Hsp70 expression. © 2005 Wiley Periodicals, Inc. J Neurobiol 64: 170–180, 2005

Keywords: heat-shock; excitatory junctional potentials; natural thermotolerance; synaptic plasticity; *Drosophila*

INTRODUCTION

The protection of neuronal transmission is critical for survival. Animals are regularly challenged by capricious or severe environments such as inclement weather or extreme temperatures. While it is known that vertebrates and invertebrates alike have evolved highly effective behavioral and physiological strategies to cope with severe environments (for reviews see Armstrong and Stoppani, 2002; Wingfield, 2003), very little is known about how the nervous systems of organisms are adapted to these conditions. Determining these adaptations is essential for understanding how organisms survive in harsh environments because neuronal circuits will fail and imperil an animal long before tissues and cells begin to die. Environments with extreme temperatures, hot deserts for example, are especially interesting because in the face of high temperature stress synaptic failure may be the primary cause of death (Hochachka and Somero, 2002).

The acute thermosensitivity of synapses is demonstrated by reliable decreases in the amplitude and

duration of the synaptic potential at the neuromuscular junction in response to increased temperature (Katz and Miledi, 1965). A decrease in amplitude and duration with extreme temperature may indicate several affected processes, such as an alteration in transmitter release, a decrease in membrane resistance, or modifications to synaptic ion channels. The molecular mechanisms of adaptation to heat stress are virtually unknown, however, knowledge of the biochemical specializations that enable nervous system function at high temperatures is pertinent to understanding synaptic plasticity in the face of natural stress.

In general, stress is defined as a condition that disrupts normal biological function or decreases fitness, in particular, cellular stress may result in detrimental changes in protein conformation and/or harmful proteinaceous aggregations (Parsell and Lindquist, 1993). Protection against cell death during temperature stress has previously been attributed to the up-regulation of heat-shock proteins (HSPs) (Burton et al., 1988) in what is known as the "heat-shock response," a protective effect stimulated by prior stress exposure. HSPs, which act as molecular chaperones to preserve cellular integrity, promote the reacquisition or maintenance of the native protein structure and function (Hartl, 1996). Further studies have demonstrated that exposure to prior heat-shock results in neuronal thermoprotection (reviewed in Yenari, 2002). More specifically, prior heat-shock protects against failure in the locust flight circuit (Robertson et al., 1996), maintains the integrity of synaptic transmission in both *Drosophila* (Karunanithi et al., 1999) and the mammalian brainstem (Kelty et al., 2002), and prevents experimental suppression of synaptic plasticity in the rat (Lin et al., 2004).

HSPs are highly conserved and ubiquitous among taxa. For example, in rat nervous tissue Hsp40 has been localized in postsynaptic sites, and Hsc70, a constitutive form of Hsp70, plays a role in clathrin uncoating in the presynaptic endocytotic vesicular process (Ohtsuka and Suzuki, 2000). In human development, Hsp70 is an antiapoptotic factor (Christians et al., 2003), and in *Drosophila melanogaster* Hsp70 expression is rapidly induced during thermal stress and plays a role in synaptic protection (Karunanithi et al., 2002). Nevertheless, for animals inhabiting hot environments little is known of the natural mechanisms protecting synaptic function. There is evidence, however, that the up-regulation of HSPs does not play a role in natural thermoprotection. Analysis of thermoresistant strains of *Drosophila* has revealed no correlation between HSP expression and high thermotolerance (Molodtsov et al., 2001; Sorensen et al., 2001). Notably, Krebs (1999) reported that the temperature that maximally induced Hsp70 expression was a poor inducer of thermotolerance and that high

levels of Hsp70 harmed, rather than protected, some southern *Drosophila* species. In *D. melanogaster*, Hsp70 expression sensitized development to thermal damage (Williams et al., 2003). Furthermore, several studies have demonstrated a detrimental effect of HSP over-expression on growth, development rate, and fertility (Krebs and Feder, 1997; Sorensen et al., 2003).

Many experiments have tested thermotolerance in *Drosophila* (examples above), however, these studies focused on standard lab-reared species (i.e., *D. melanogaster*), and moreover they evaluated performance by measuring development (Williams et al., 2003), longevity (Le Bourg et al., 2001), knockdown resistance (McColl et al., 1996), and fecundity (Hercus et al., 2003). While these measures are useful in providing information regarding overall fitness, the underlying neurophysiology that governs behavior may better demonstrate fundamental thermoresistance. Using two ecologically distinct species of *Drosophila* we first determined that thermal stress differentially affected the locomotor behavior of unrestrained intact larvae. We then investigated differences in the thermosensitivity of the underlying neurophysiology mediating locomotory behavior and whether prior heat-shock exposure afforded neuroprotection to the neuromuscular junction (NMJ) in both species.

The adaptation of *Drosophila* to thermal extremes has been well studied and is a widely used model for studying ecological responses to extreme temperatures (Maynard-Smith, 1956; Hosgood and Parsons, 1968; Patton and Krebs, 2001). Knowing that high temperatures damage organismal physiology, particularly neural transmission, elucidation of the mechanism of synaptic thermotolerance is important to understand diseases related to thermal stress, such as heat-stroke and sudden infant death syndrome. Furthermore, understanding differences in naturally thermotolerant organisms is important not only for understanding the adaptive life histories of poikilothermic organisms living in hot environments, but for understanding the protective mechanisms of nervous system function during ecologically relevant stress.

Drosophila arizonae, which diverged from *D. melanogaster* approximately 55–65 million years before present (Gibbs and Matzkin, 2001; Staten et al., 2004), inhabit desert cacti in the southwestern United States of America. The larvae and pupae regularly experience temperatures exceeding 40°C (Patton and Krebs, 2001), which makes the desert fly a perfect model for investigation of thermal stress on neural transmission. *D. melanogaster*, on the other hand, have had a long history of continuous culture in a narrow temperature range (22–25°C) and provide an ideal species for comparison. In this study, we

evaluated differences in synaptic thermosensitivity between species to identify potential underlying physiological mechanisms of natural thermoprotection at the synapse.

MATERIALS AND METHODS

Fly Stocks

Two species of *Drosophila* were used in this study. The common wild-type strain of *D. melanogaster* larvae, Canton-S, was obtained from the Queen's University population and raised on a standard molasses medium at room temperature (20–23°C). *D. arizonae,* a cactophilic species native to the Sonoran desert, were ordered from FlyBase at the Tucson stock center (stock #: 15081-1271.04) and maintained at Queen's University on a banana medium at room temperature for 4 weeks prior to commencing experiments to ensure that the recent thermal histories for both species were similar. Maximum and minimum temperatures in the Sonoran desert, 47 and −8.9°C respectively, were far more variable than rearing temperature for *D. melanogaster* (approximately 22°C). Temperature data on monthly averages of the maximum and minimum temperatures were provided for the Sonoran Desert by Jackson et al. (1991).

Heat-Shock

Larvae receiving a heat-shock treatment were placed in a standard Petri dish containing tissue paper moistened with distilled water and sealed shut to preserve humidity. Larvae were heat-shocked at 36°C for 1 h, then allowed to recover in the dish at room temperature for 1 h before electrophysiological experimentation and Western blot analysis. In addition, a group of *D. arizonae* larvae were heat-shocked at 40°C and allowed to recover at room temperature for 1 h before Western blot analysis.

Kinematic Analysis

Third instar *D. melanogaster* ($n = 9$) and *D. arizonae* ($n = 8$) larvae were placed on a metal dish (6 cm diameter) containing 30 mL of 1.3% hardened non-nutritive agar solution. The temperature of the agar was increased at a rate of 3°C per minute from 20°C to the temperature at which locomotion ceased (40–50°C). The temperature was controlled using a Peltier Plate upon which the dish was placed. An IT-23 copper-constantan thermocouple (Physitemp Instruments, NJ) was used to monitor and record temperature. Prior to the experiment, larvae were gently brushed with a small amount of nontoxic black ink in order to visualize individual segments.

Locomotion was filmed at 5°C intervals using a CCD camera mounted on a stereomicroscope (PZMTIII; World Precision Instruments) and was recorded in VHS format. Videos were digitized using a video-capture board at a sample rate of 10 frames per second. Digitized files were analyzed using Videopoint (2.5) motion analysis software (LSW Software, MA). Crawling frequency was calculated by measuring the time period between peaks in the displacement pattern of the head segment and then compared between species.

Semi-Intact Preparation

During dissection third instar larvae were bathed in zero-calcium hemolymph-like solution (HL3; Stewart et al., 1994). Animals were pinned using fine insect pins onto a glass plate. Heart and viscera were removed then the segmental nerve roots were severed and the central nervous system was also removed. The filleted larvae contained only body wall, body wall muscles, and severed nerves (as described by Jan and Jan, 1976). Experiments were conducted in an HL3 superfusion containing calcium (1.5 m*M*). Muscle size was quantified by measuring the area of the internally facing muscle fiber 6, which is a good index of muscle surface area as the muscles are flat.

Electrophysiology

Intracellular recordings of stimulated excitatory junctional potentials (EJPs) were made with glass microelectrodes (filled with 3 *M* KAc; 25–60 MΩ) from ventral abdominal muscle 6 in segment A3. Quantal size was estimated by calculating the average amplitude of spontaneous miniature EJPs for a given temperature and quantal content was estimated by calculating the ratio of the averaged EJP amplitude to the averaged mean quantal size at a given temperature. Input resistance was measured using two intracellular electrodes positioned in muscle m6 of segment A3; one electrode injected a square current pulse while the other recorded the change in membrane potential. To evaluate effects of thermal stress on the EJP, a saline-filled suction electrode was used to stimulate suprathreshold, the severed end of the motor nerve with square voltage pulses (duration: 0.4 ms; frequency: 0.4 pps) thus ensuring that failure of the EJP was not a result of failure to stimulate an event. Recordings of EJPs were made as temperature of the HL3 perfusion was increased by approximately 3°C per minute from 25 to 40°C, at which point temperature was held at 40°C until EJPs were no longer being stimulated (characterized as failure). The temperature of the preparation was increased using a PTOC Proportional Temperature Controller (Scientific Systems Design, Toronto, ON, Canada). The preparation was viewed with a 10X lens using Olympus optics and images were magnified a further 10X using a CCD camera to aid in visualization. EJPs were recorded and signals were amplified using a Neuroprobe 1600 amplifier (A-M Systems Inc., PA) and then digitized using a Digidata 1322A (Axon Instruments, Foster City, CA). Signals were acquired using the pClamp 9.0 software package.

Western Blot Analysis of Hsp70

A Western blot was preformed to determine if *D. arizonae* had higher Hsp70 expression. Non-heat-shocked and heat-
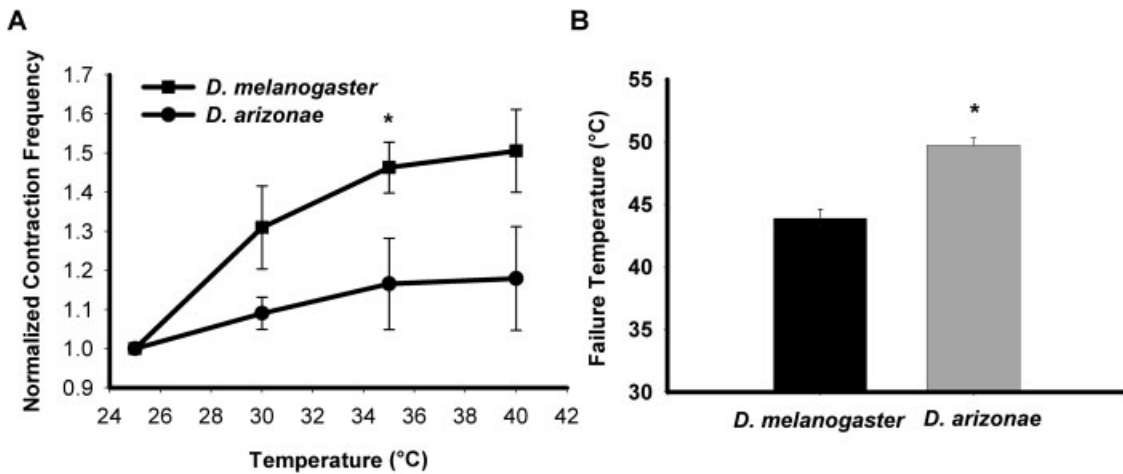
Figure 1   Increasing the temperature of the substrate affects locomotory behavior in unrestrained *Drosophila* larvae (*D. melanogaster*, $n = 9$; *D. arizonae*, $n = 8$). (A) Contraction frequency of *D. melanogaster* was significantly more thermosensitive. (B) *D. arizonae* are able to locomote at significantly higher temperatures. Data are means $\pm$ SE, and an asterisk indicates significant difference between species.

shocked third instar larvae from *D. arizonae* and *D. melanogaster* lines were collected, frozen in liquid nitrogen, and stored at -80°C. Groups of 10 larvae were homogenized in 110 μL of ice-cold extract buffer (10 m*M* Tris-Cl, pH 8.0, 50 m*M* NaCl, 1% NP-40) with protease inhibitor (Roche Complete, Cat #1697498, at the concentration of 1 tablet/50 mL) in 1.7 mL microfuge tubes with a Teflon pestle. The homogenates were centrifuged at 13,000 $\times$ *g* for 10 min at 4°C and the supernatants then drawn off and preserved on ice. The total protein concentrations (μg/μL) were quantified using the Bio-Rad protein assay (Cat # 500-0006). The samples were denatured by heating at 100°C for 5 min with an equal volume of 2 $\times$ SDS sample buffer (125 m*M* Tris, pH 6.8, 4.6% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.1% bromophenol blue). SDS-PAGE was performed on 10% gels with a 5% stacking gel using the discontinuous buffer system of Laemmli (1970). Proteins (μL, calculated from assay) were transferred onto a nitrocellulose membrane in transferring buffer (25 m*M* Tris, 192 m*M* glycine, 20% methanol). Blots were then stained with Ponceau S (P-7170; Sigma) to ensure equal loading of protein in all lanes.

For Western analysis of Hsp70 protein, blots were washed for 10 min in 0.1 *M* PBS, pH 7.4, and the background was saturated for 1 h at 37°C in block buffer (5% skim milk in 0.1 *M* PBS) and then incubated overnight at 4°C in primary antibody (MA3-007, 1:1000 in block buffer; Affinity Bioreagents). MA3-007 is recombinant mouse antihuman Hsp70 monoclonal antibody and reacts in several species, including *Drosophila*. The 70 kDa Hsp70 family contains two main members, the inducible Hsp70 (HSP70) and constitutive Hsp70 (HSC70). In addition, Hsp70 family is highly conserved in all organisms and the broad specificity of the antibody makes it appropriate for both *Drosophila* species. Blots were then washed three times for 5 min each in TBS (0.1 *M*

PBS with 0.05% Tween 20) and then incubated for 2 h at 37°C in horseradish peroxidase (HRP)-conjugated secondary antimouse IgG antibody (SAB-100, 1:5000 in block buffer; StressGen). Blots were again washed three times for 5 min each and a fourth time for 10 min in TBS. An enhanced chemiluminescence kit (RPN2135; Amersham) was used to visualize the immunoreaction. Bands were photographed and quantified using SynGene GeneTools Analysis Software (Version 3.03.03; SynGene).

Statistical Analysis

Crawling frequency and locomotory failure temperature data files were transferred to SigmaStat 3.0 (Jandel Scientific, San Rafael, CA) and differences between species were analyzed using two-tailed *t* tests. The pClamp files were analyzed in Clampfit 9.0 and raw data were compiled and analyzed using commercial software (Sigmaplot 8.0; Jandel Scientific). EJP amplitude and duration were analyzed at 5° intervals from 25–40°C for animals receiving no heat-shock (control) and animals receiving heat-shock (heat-shocked) for both species. The effects of thermal stress on EJP characteristics and time to synaptic failure at 40°C were assessed using nine *D. melanogaster* controls, nine *D. melanogaster* heat-shocked, nine *D. arizonae* controls, and ten *D. arizonae* heat-shocked. Statistical significance was tested using appropriate parametric or nonparametric tests as stated in the text. Statistical analyses were performed using SigmaStat 3.0 and significance was assessed at $\alpha < 0.05$. To assess differences between the four groups (control and heat-shocked groups in both species), the one-way ANOVA test was used at each 5°C interval. Pairwise comparisons between all groups were made using the Holm-Sidak test, and between two groups using a *t* test.

Table 1   **EJP Amplitude and Duration for Control (C) and Heat-Shocked (HS) Preparations of**
***D. melanogaster* (DM) and *D. arizonae* (DA) during an Increase in Internal Temperature from 25°C to 40°C**

	DM C	DM HS	DA C	DA HS
EJP Amplitude (mV)				
25° C	24.93 ± 2.70	26.25 ± 3.37	20.86 ± 1.8	24.97 ± 2.68
30° C	22.04 ± 2.70	26.67 ± 3.94	21.66 ± 1.45	25.72 ± 2.56
35° C	20.40 ± 2.36	22.55 ± 2.88	21.60 ± 1.63	23.69 ± 1.78
40° C	8.58 ± 1.88	12.42 ± 1.69	15.57 ± 1.89	17.62 ± 1.80
EJP duration (ms)				
25° C	15.76 ± 1.99	21.20 ± 3.03	19.89 ± 1.48	12.84 ± 1.94
30° C	8.35 ± 1.88	15.90 ± 2.60	18.49 ± 1.75	12.09 ± 1.63
35° C	6.78 ± 1.19	12.36 ± 1.61	14.51 ± 1.28	10.68 ± 0.84
40° C	7.72 ± 3.33	10.05 ± 0.56	12.33 ± 1.52	8.29 ± 1.42

Data are means ± SE.

The mean ± standard error is given as the measures of central tendency and variability, respectively.

RESULTS

Thermosensitivity of Locomotor Behavior

As temperature of the substrate was increased, segmental contraction frequency of the larvae increased in both species. Contraction frequency in *D. melanogaster* (DM) was significantly more thermosensitive at higher temperatures [Fig. 1(A)] (35°C: t test, $t = 2.36$, $df = 14$, $p < 0.05$), a trend that was also reflected at 30 and 40°C. The temperature at which contractions failed was significantly higher for the desert species, *D. arizonae* (DA) [Fig. 1(B)] ($t = -5.99$, $df = 15$, $p < 0.001$).

Thermosensitivity of EJPs

The results of thermal stress on untreated (Control: C) and heat-shocked (HS) preparations of both species are presented simultaneously in the figures but the controls are described first in the text. As the internal temperature of a dissected larva was increased, EJP amplitude (mV) and duration (ms) decreased in both species (Table 1). At high temperatures relative EJP amplitude decreased less in *D. arizonae* than in *D. melanogaster* (35°C: $t = -2.579$, $df = 15$, $p < 0.05$; 40°C: $t = 4.60$, $df = 15$, $p < 0.001$) [Fig. 2(A,B)]. Absolute EJP duration appeared to be larger in *D. arizonae* at room temperature (Table 1) but was not significant ($p = 0.107$). Nevertheless, EJPs were more thermosensitive in *D. melanogaster*, demonstrated by a greater effect of an increase in temperature from 25 to 30°C on duration (DM: $t = 5.80$, $df = 15$, $p < 0.001$; DA: $df = 15$, $p > 0.05$) [Fig. 2(C)]. Relative EJP duration at half-ampli-

tude decreased less in *D. arizonae* during intermediate internal temperatures (30°C: $t = -4.08$, $df = 15$, $p < 0.001$; 35°C: $t = 4.39$, $df = 15$, $p < 0.001$), but was not significantly different between species at 40°C [Fig. 2(C)].

To determine whether the species-specific differences were localized to the pre- or postsynaptic membrane, quantal size and quantal content were analyzed from 25–35°C in both species. There was no significant difference in quantal size or quantal content between species and no significant effect of temperature within a species, although there was a trend suggesting that the mean quantal size was smaller in *D. arizonae* (35°C: $p = 0.055$) and there was a corresponding increase in quantal content (Fig. 3).

To explore the nature of synaptic failure at high temperatures, EJP transmission was monitored in both species at 40°C for a maximum of 20 min. At high temperatures, *D. arizonae* continued to display EJPs for significantly longer than *D. melanogaster* [Fig. 4(A)] (Mann-Whitney Rank Sum Test, $t = 132$, DM: $n = 9$, DA: $n = 9$, $p < 0.001$).

Any effect of temperature on synaptic properties could be a consequence of differences in the electrophysiological properties of the postsynaptic membrane. Thus it is necessary to determine whether there are species-specific differences in the intrinsic membrane properties. Input resistance was significantly higher in *D. melanogaster* ($n = 6$) than *D. arizonae* ($n = 4$) across all temperatures (Fig. 5) [two-way repeated measures ANOVA, $F(3, 21) = 8.$; $p < 0.001$]. Finally, there was no appreciable between-species difference in larval size and this was supported by measurements of the inside facing surface area of m6 in segment A3 of *D. arizonae*, which did not differ significantly from the area of the same muscle in *D. melanogaster* as previously published by Li et al. (2002) for late third instar larvae (28–45 × 10^3 μm^2). In addition, there was no
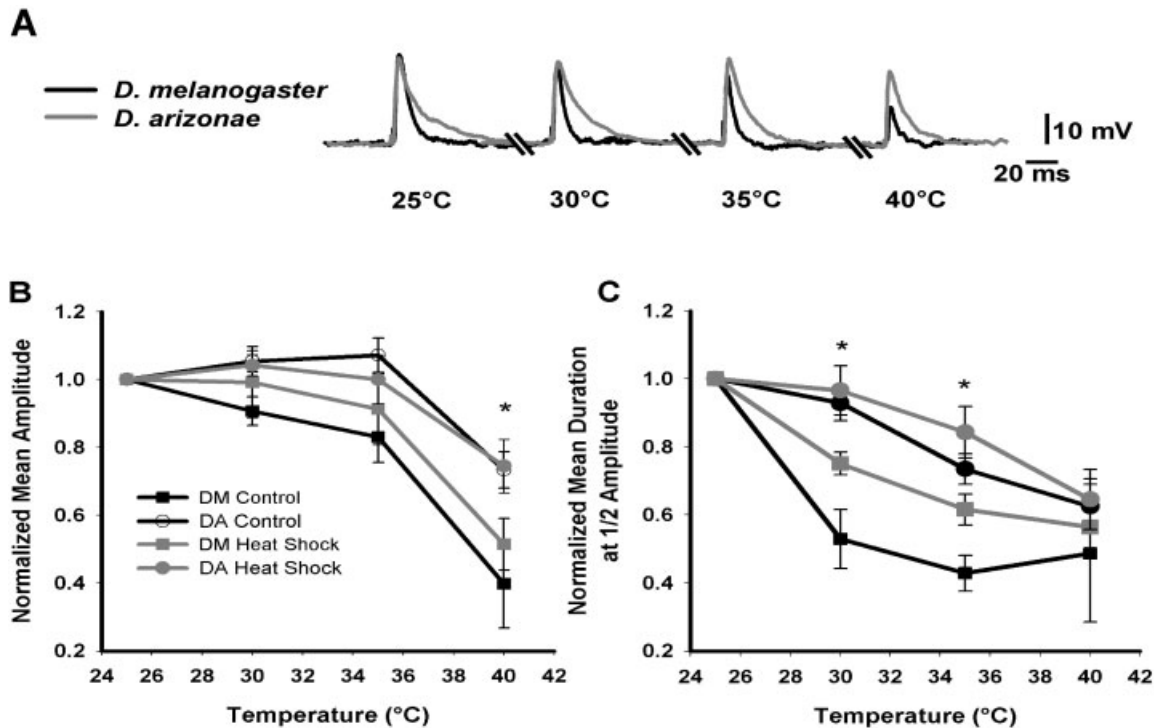
Figure 2 The effect of increasing temperature on (A) EJP activity of a filleted untreated *Drosophila* preparation during a heat ramp, (B) relative EJP amplitude, and (C) relative duration in control and heat-shocked preparations of *D. melanogaster* (DM) and *D. arizonae* (DA). (A) EJP profiles monitored by intracellular recordings from muscle A3. EJPs are shown at 25, 30, 35, and 40°C of control (non-heat-shocked) preparations of both *D. melanogaster* (black) and *D. arizonae* (grey). Breaks in traces indicate a break in the time scale as temperature was being increased. (B) Relative EJP amplitude is greater in *D. arizonae* at 40°C and heat-shock had no effect on amplitude in either species. (C) Relative duration was significantly greater in *D. arizonae* and heat-shock significantly increased the relative duration in *D. melanogaster*. Data are means ± SE, and an asterisk denotes significant difference in (B) DA Control and DA Heat-Shock from DM Control; (C) DA Control, DA Heat-Shock, and DM Heat-Shock from DM Control.

significant difference in resting membrane potential between the two species.

To determine whether a prior heat-shock affords protection to both the control and the naturally thermotolerant desert species, EJP characteristics were evaluated in heat-shocked preparations of both species (Table 1). *D. melanogaster* experienced thermoprotective effects of prior heat-shock, whereas *D. arizonae* did not. However, in *D. arizonae* there was a significant difference in quantal size and quantal content following a heat-shock but this difference was expressed at 35°C only (quantal size: Mann-Whitney Rank Sum Test: $t = 10.0$, DA: $n = 4$, DM: $n = 6$, $p < 0.01$; quantal content: $t = 3.616$, $df = 8$, $p < 0.01$) (Fig. 3).

In *D. melanogaster,* heat-shock pretreatment affected EJP thermosensitivity, demonstrated by a significant increase in relative EJP duration from that of the control (30°C: $t = -3.689$, $df = 14$, $p < 0.01$; 35°C: $t = 2.70$, $df = 17$, $p < 0.05$) [Fig. 2(C)]. Time

required for EJP transmission to fail at high temperatures was significantly increased in *D. melanogaster* ($t = -2.19$, $df = 17$, $p < 0.05$), but not in *D. arizonae* [Fig. 4(A)], and there remained no significant difference in relative EJP amplitude across both treatment groups in both species at 40°C. Prior heat-shock also altered the incidence of EJP recovery only in *D. melanogaster*. Significantly fewer (22%, 2/9) control *D. melanogaster* recovered, whereas (89%, 8/9) prestressed animals recovered (z test for pairwise comparisons of proportionate data; $z = 2.369$, $p < 0.05$) [Fig. 4(B)].

The induction profile of Hsp70 in whole-body lysates of both species parallels the electrophysiological results. Following 1 h heat-shock treatment at 36°C, a robust induction of Hsp70 was apparent in *D. melanogaster* only [Hsp70 level measured as integrated optical density (IOD): DM C = 8,764.24; DM HS 36°C = 30,956.48; DA C = 25,295.09; DA HS 36°C = 23,921.67; DA HS 40°C = 20,718.74]
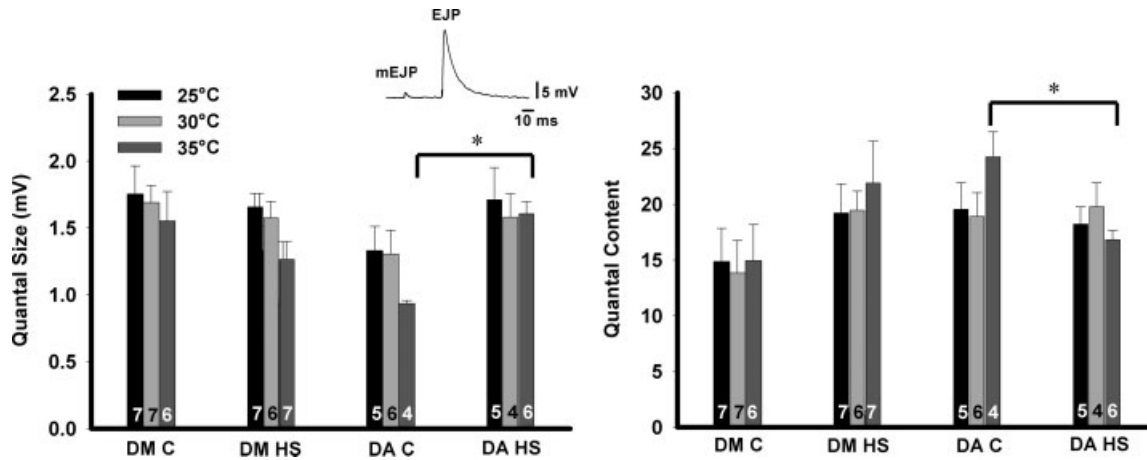
Figure 3 Quantal size (left) and quantal content (right) for control (C) and heat-shocked (HS) preparations of *D. melanogaster* (DM) and *D. arizonae* (DA) as internal temperature was being increased. Numbers within bars indicate number of individuals in the group. Data are means ± SE, and asterisk indicates significant difference between control and heat-shocked preparations at 35°C. Top inset demonstrates the miniature EJP (mEJP).

(Fig. 6). To control for the possibility that the heat-shock treatment temperature was simply not stressful enough for the induction of Hsp70 in the desert species, the induction profile was analyzed for *D. arizonae* following 1 h heat-shock treatment at 40°C with no increase in Hsp70 expression (Fig. 6).

DISCUSSION

This study confirmed that thermosensitivity of nervous system function differs significantly between ecologi-

cally distinct species. Natural thermoprotection in the desert species, *D. arizonae*, was demonstrated by an increased upper temperature limit for larval locomotion and also by maintained integrity of synaptic transmission during high temperature stress. Western blot analysis revealed higher levels of inducible Hsp70 in *D. arizonae* at control temperatures; however, there was no increase in Hsp70 expression following heat-shock, whereas there was almost a threefold increase in Hsp70 expression in *D. melanogaster* after heat-shock. Finally, prior heat-shock afforded neuronal pro-
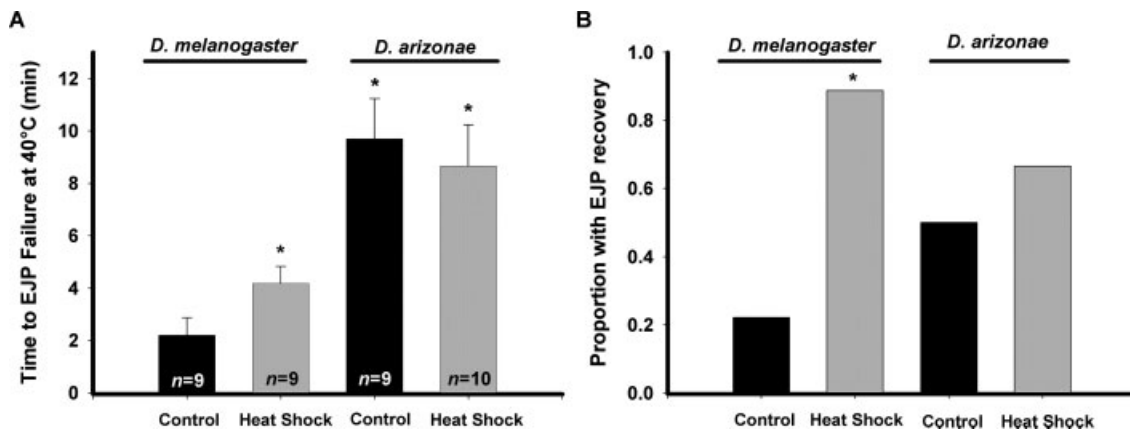

Figure 4 (A) During exposure to sustained heat application (internal temperature maintained at 40°C), EJP transmission continues for longer in control preparations of *D. arizonae* than in *D. melanogaster* (black bars). Prior heat-shock increases time to EJP failure in *D. melanogaster* only (gray bars). There was no significant difference between control and heat-shocked *D. arizonae*, however, synaptic transmission in control and heat-shock preparations continued for longer than both control and heat-shock preparations of *D. melanogaster*. (B) Subsequent to heat-induced EJP failure, proportion of animals that experienced recovery of EJPs within 20 min of failure. Heat-shock increased the proportion of recovery in *D. melanogaster*. Data are means ± SE, and asterisk indicates significant difference from *D. melanogaster* control.
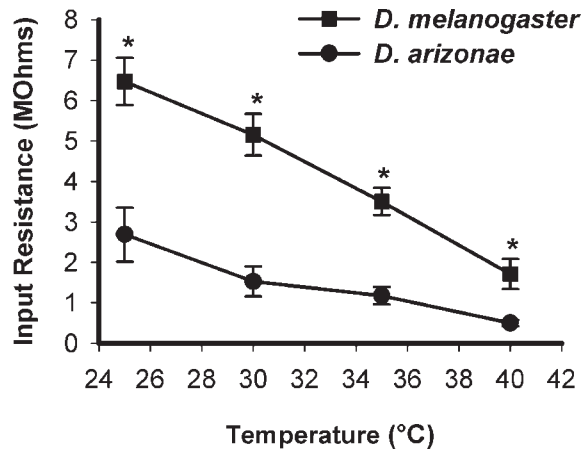
Figure 5 Input resistance, recorded from muscle m6 in segment A3, was significantly higher in *D. melanogaster* ($n = 6$) than in *D. arizonae* ($n = 4$). Data are means $\pm$ SE, and asterisks denote significant difference between species at each temperature.

tection only to the temperate species, although the desert species had a higher baseline thermotolerance than both control and heat-stressed temperate organisms. These data support the hypothesis that organisms native to hot environments are adapted to high temperatures by having higher baseline HSP levels.

Thermosensitivity

Thermosensitivity of locomotor behavior was demonstrated in both species by an overall increase in segmental contraction frequency with a corresponding increase in temperature. This reaction has been previously documented (Chu et al., 2003) and can be functionally explained as a behavioral means to control body temperature by attempting to avoid hot microenvironments that would otherwise threaten survival (e.g., Schultz, 1998). The desert species, *D. arizonae*, was significantly more tolerant to a temperature increase, and an interesting question is how the neurobiology of this organism differs from *D. melanogaster* in order to maintain function at high temperatures.

In both species EJPs at the neuromuscular junction were thermosensitive. These results are consistent with previous research regarding the effects of temperature on postsynaptic potentials (reviewed in Janssen, 1992; Robertson, 1993). In *D. arizonae*, EJPs were less thermosensitive than in *D. melanogaster*, moreover the effects of temperature on the EJP were manifest at cooler temperatures for *D. melanogaster* and relative EJP duration was greater at all temperatures in *D. arizonae*. These results are similar to those of Rosenthal and Bezanilla (2002), who observed comparable results for propagated action potentials from tropical and tem-

perate squid species. They proposed action potential broadening as an adaptation to avoid failure at high temperatures in species from warm environments. Similarly, broadening of synaptic potentials may facilitate maintained synaptic transmission and protect against failure at high temperatures in *D. arizonae*. Because the duration of the synaptic potential was greater in the desert species, it may follow that the input resistance (Rm) of the muscle fiber would be higher than in the temperate species, assuming that capacitance and other parameters remain constant. Contrary to expectations, however, we found input resistance of the muscle fiber in the desert species to be significantly lower and less affected by temperature. These results suggest that the synaptic differences are not merely confined to the passive properties of the muscle membrane.

EJP Failure at High Temperatures

In the present study, EJPs in a desert species were more thermotolerant, displayed in part by an increased time to failure at high temperature. Prior studies have induced thermotolerance in *Drosophila* (Cavicchi et al., 1995; Dahlgaard et al., 1998) and invoked an up-regulation of HSP expression as the thermoprotective mechanism. Specifically, in *D. melanogaster* prior heat-shock improved synaptic performance at the neuromuscular junction during heat stress (Karunanithi et al., 1999) and protection has subsequently been attributed to Hsp70 (Karunanithi et al., 2002; Brown et al., 2004). Similarly, in our study, prior heat-shock had a protective effect on EJP
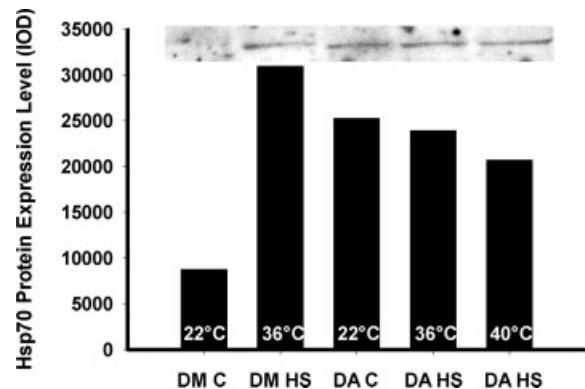

Figure 6 Under control conditions, Hsp70 expression is greater in *D. arizonae*. Heat-shock at 36°C for 1 h induced Hsp70 protein expression in *D. melanogaster* (DM) but not in *D. arizonae* (DA). Wandering third instar larvae were exposed to either a 36 or a 40°C heat-shock and allowed to recover for 1 h at 22°C. Values presented are measures of integrated optical density (IOD). Numbers in bars indicate the control condition (22°C) or the heat-shock temperature (36 or 40°C).

production during subsequent prolonged heat exposure in *D. melanogaster*, however, there was no protective effect observed in the desert species, *D. arizonae*. Synaptic protection in *D. melanogaster* was demonstrated by a 1.89-times increase in time to EJP failure at high temperatures and a doubling of the proportion of larvae that recovered EJP activity following heat-induced failure. The temporal effects, such as an increase in the time to failure and increased proportion of animals recovering after failure at high temperature, have been demonstrated previously in motor pattern generation in the locust (Newman et al., 2003) and suggest a time-dependent increase in protein damage that is mitigated by heat-shock induced protection. Thus, the induction of molecular chaperones, Hsp70 for example, may extend the functional range of neuronal processes.

Despite a lack of induced thermoprotection in *D. arizonae*, synaptic transmission in non-heat-shocked preparations was more robust than in *D. melanogaster*. EJPs continued approximately 4.4 times longer than that of the untreated *D. melanogaster* and for approximately 2.3 times longer than that of *D. melanogaster* exposed to prior heat-shock. It is tempting to attribute this natural thermotolerance in *D. arizonae* to increased constitutive expression of Hsp70 as previously demonstrated for lizards (Ulmasov et al., 1992) and ants (Gehring and Wehner, 1995). We were able to test this hypothesis by Western blot analysis of Hsp70 and we found that baseline Hsp70 expression was significantly higher in *D. arizonae*. Interestingly, there was no increase in Hsp70 expression following heat-shock in *D. arizonae*, however expression in *D. melanogaster* increased threefold. It is possible that at temperatures greater than 40°C inducible Hsp70 plays a greater role in thermoprotection for *D. arizonae*, nevertheless we were interested in what mediates thermoprotection under the natural conditions to which this species is regularly exposed. However, an interesting future direction would investigate synaptic thermotolerance at temperatures above *D. arizonae's* natural range. The Western analysis suggested that Hsp70 might be involved in neuronal thermoprotection in *D. arizonae*, which is consistent with data reported for the African migratory locust (Qin et al., 2003). In contrast, several studies have demonstrated the deleterious consequences of long-term over-expression of HSPs in *Drosophila* (Krebs and Feder, 1997; Bettencourt et al., 1999), and it is possible that at temperatures warm enough to induce HSP expression but insufficient to kill *Drosophila* rapidly, HSPs may have deleterious effects, such as reduced fecundity (Krebs and Feder, 1997) and retarded development (Feder et al., 1992). Therefore, *D. arizonae* may not have experienced a classic "heat-shock response" when exposed to prior heat treatment because in a hot environment it may be too costly to initiate the heat-shock response constantly; however, they have adapted to high temperatures by expressing a higher baseline level of Hsp70.

Alternative Mechanisms of Protection

An alternative mechanistic explanation, which is not mutually exclusive with the above, involves species-specific changes in synaptic morphology that may provide organisms in extreme environments with a broader temperature range over which functional properties are stabilized. Interestingly, quantal size in *D. arizonae* was slightly smaller than in *D. melanogaster* and there was a corresponding increase in quantal content. It would be expected with a lower input resistance in *D. arizonae* that the quantal size would also be smaller because with a given excitatory junctional current a low Rm in the desert species will result in a smaller quantal size. Given the highly significant difference in Rm, therefore, one would predict the quantal size to be significantly different. Thus, postsynaptic modifications such as differences in synaptic area or in postsynaptic receptors available for activation may play a role in natural thermoprotection in the desert species. Also, presynaptic morphology may differ across species and future studies investigating neuromuscular innervation patterns may provide evidence of adaptive change in bouton number and/or size.

CONCLUSION

This study provides evidence that species originating from stressful environments have evolved neural adaptations that better enable synaptic transmission at high temperatures. There was a significant difference in the time required for transmission of a desert species' EJPs to fail while their internal temperature was being held at a sublethal temperature. This significant difference in neural activity reflected the significant difference between species in the temperature at which whole animal locomotory behavior failed. Exposure to a prior heat-shock afforded significant thermoprotection to synaptic circuitry in only the temperate species, and Western analysis of Hsp70 levels confirmed that an up-regulation of Hsp70 occurred in *D. melanogaster* only. Western analysis also revealed that the increased thermotolerance of *D. arizonae* is associated with a higher baseline expression of Hsp70, although Hsp70 expression was greater in *D. melanogaster* following heat-shock. These data, when taken together, suggest

that differences in ecology may demand different thermoprotective mechanisms, such as variation in pre- and postsynaptic properties and/or an increase in baseline expression levels of Hsp70. Investigations into stress-mediated thermoprotection in naturally thermotolerant *Drosophila* will be a viable and profitable research avenue for discovering the subcellular mechanisms of this neural plasticity.

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