Role for Calcium in Heat Shock-Mediated Synaptic Thermoprotection in *Drosophila* Larvae

J.W. Barclay,* R.M. Robertson

Department of Biology, Queen's University, Kingston, ON, Canada K7L 3N6

ABSTRACT: Chemical synaptic transmission is the mechanism for fast, excitation-coupled information transfer between neurons. Previous work in larval *Drosophila* has shown that transmission at synaptic boutons is protected by heat shock exposure from subsequent thermal stress through pre- and postsynaptic modifications. This protective effect has been, at least partially, ascribed to an up-regulation in the inducible heat shock protein, hsp70. Effects of hsp70 are correlated with changes to intracellular calcium handling, and the dynamics of intracellular calcium regulate synaptic transmission. Consistent with such a relationship, synaptic plasticity increases at locust neuromuscular junctions following heat shock, suggesting an effect of heat shock on residual presynaptic calcium. Intracellular recording from single abdominal muscle fibers of *Drosophila* lar-vae showed that prior heat shock imparts thermoprotection by increasing the upper temperature limit for synaptic transmission. Heat shock exposure enhances short-term synaptic plasticity and increases its thermosensitivity. Increasing extracellular calcium levels eliminates the physiological differences between control and heat shock preparations; excess calcium itself induces thermoprotection at elevated concentrations. These data support the hypothesis that stress-induced neuroprotection at the nerve terminal acts, at least partially, through an alteration to the physiological effects of residual presynaptic calcium. © 2003 Wiley Periodicals, Inc. J Neurobiol 56: 360–371, 2003

Keywords: calcium; *Drosophila*; heat shock; synaptic plasticity; thermotolerance

INTRODUCTION

Pre-exposure to elevated temperature (heat shock) has been demonstrated to protect cells, tissues, and organisms during subsequent stressful events (Parsell and Lindquist, 1993; Estruch, 2000; Jolly and Morimoto, 2000). This response has been classically attributed to an up-regulation in the expression of heat shock proteins, cellular protein chaperones acting to maintain protein conformation and function during stress and to prevent the formation of nonfunctional protein aggregates (Craig et al., 1994; Fink, 1999; Hartl and Hayer-Hartl, 2002). The heat shock response is evident in the nervous system, where it improves the survival rate of cells, synapses, and tissues during stressful conditions (Mailhos et al., 1993; Fink et al., 1997; Ohtsuka and Suzuki, 2000) and has been identified as a novel neuroprotectant in several experimental models of nervous system injury (Yenari et al., 1998, 1999). Recent work has shown that prior heat shock not only enhances survival rate, but in fact confers a functional neuroprotection at synapses (Dawson-Scully and Robertson, 1998; Karunanithi et al., 1999; Barclay and Robertson, 2000, 2001; Kelty et al., 2002) that is advantageous for preservation of critical behavioral responses (Barclay and Robertson, 2000). The underlying cellular/molecular mechanisms involved, however, remain unknown.

*Present address: Physiological Laboratory, University of Liverpool, Crown St., Liverpool, UK L69 3BX.

Correspondence to: R. M. Robertson (robertrm@biology.queensu.ca).

Heat shock increases short-term synaptic facilitation at the hindleg neuromuscular junction of the locust (Barclay and Robertson, 2001); this is likely to be accomplished through an effect on residual presynaptic calcium levels during high-frequency synaptic transmission (Fisher et al., 1997; Zucker, 1999). The onset and duration of heat shock-induced protection of presynaptic function mirrors those for the up-regulation of hsp70 expression (Karunanithi et al., 1999). As well, transgenic larvae with increased hsp70 expression demonstrate enhanced levels of presynaptic protection (Karunanithi et al., 2002), and exogenous hsp70 attenuated the effects of thermal stress on synaptic parameters in mouse brainstem slice preparations (Kelty et al., 2002), supporting the hypothesis for an involvement of this cellular chaperone. Hsp70 action has also been linked with intracellular calcium handling by a number of biochemical and physiological studies (Smith et al., 1995; Kiang et al., 1998; Nánási et al., 2000; Torrecilla et al., 2000). To test whether heat shock affects short-term synaptic plasticity through an effect on presynaptic calcium, we investigated extracellular calcium concentration and the heat shock response at the *Drosophila* neuromuscular junction during repetitive presynaptic stimulation.

Using intracellular recording to monitor EJPs in single muscle fibers of larval *Drosophila*, we show that exposure to prior heat shock imparts thermoprotection by increasing the functional range for synaptic transmission by 5°C. In agreement with previous results (Barclay and Robertson, 2001), we found that heat shock also enhanced the endogenous extent and thermosensitivity of synaptic plasticity during tetanic stimulation. Elevation of extracellular calcium in control preparations induced a synaptic thermoprotection similar to that induced by heat shock in other preparations. This treatment nullified the heat shock-induced increase in thermoprotection of synaptic transmission, as the thermoprotective effect of calcium was less pronounced in heat shock preparations than in controls. Therefore, heat shock has a protective effect on short-term synaptic plasticity in larval *Drosophila* that appears to act via an alteration to the physiological effects of calcium within the presynaptic nerve terminal.

METHODS

Experimental Preparation

Drosophila melanogaster of the Canton-S strain were raised on a standard cornmeal medium at 25°C (60–70% relative humidity). Physiological experiments were conducted on control and heat shock wandering third-instar larvae. Heat shock larvae were exposed to 36°C for 1 h in standard Petri dishes, containing moistened filter paper, that were taped shut to preserve high humidity. Larvae were then allowed to recover at 25°C for .5 h prior to electrophysiological experiments (Karunanithi et al., 1999).

Larvae were dissected to expose the nervous system and body wall muscles (Atwood et al., 1993). The segmental nerve roots were then severed and the central nervous system removed. Experiments were conducted in standard hemolymph-like solution (HL3; Stewart et al., 1994), varying the extracellular calcium concentration as indicated. For all experiments, recordings were initially made at room temperature (15–20°C) and then continuously as the preparation temperature was gradually increased in a ramplike manner (approximately 5°/min) until complete failure of synaptic transmission was observed. Preparation temperature was controlled by a Nichrome heating coil around an inlet pipette passing superfusing solution from a reservoir maintained at room temperature. Temperature was monitored with a copper/constantan thermocouple (0.2 mm diameter, BAT-12; Sensortek, Clifton, NJ) placed adjacent to the head of the larvae.

Electrophysiological Recordings

Intracellular recordings were made with glass microelectrodes (filled with 3 mol l^{-1} KAc; 40–60 MΩ) in ventral abdominal muscle 6, segment 3 of the larvae. An intracellular experimental approach was chosen because, despite muscle contractions, penetrations remained stable over the range of firing frequency and extracellular calcium concentration used in this study. Preparations were selected for analysis provided the intracellular penetration displayed a resting membrane potential more negative than −60 mV. The innervating motor nerve contains two motor axons (Kurdyak et al., 1994); however, over the temperature range used in this experiment the stimulus threshold differential was lost. Therefore we stimulated the severed end of the motor nerve with square voltage pulses (0.3 ms duration) suprathreshold for both motor axons, thus ensuring a consistent recording of the integrated EJP for the whole muscle.

In order to investigate the effects of heat shock on short-term synaptic plasticity of the integrated EJP, a dataset was collected where the motor nerve was stimulated at a high frequency (stimulation protocol: intraburst frequency = 40 Hz; burst repetition rate = 0.1 Hz; burst duration = 250 ms). Stimulation frequency was selected as it is within the physiological range for firing frequency of both motor axons contained within the motor nerve (Barclay et al., 2002) and reliably evoked a quantifiable short-term synaptic plasticity. To manipulate presynaptic calcium levels during short-term synaptic plasticity, datasets were collected at three discrete concentrations of extracellular calcium (1, 2, and 3 m*M*).

Analysis

For all experiments, the MacLab/4S data acquisition system (AD Instruments) was used to record electrical signals and superfusate temperature simultaneously. Digitized recordings were subsequently analyzed using the Igor Pro 3 software analysis package (Wavemetrics). For each animal, EJPs were analyzed for amplitude (measured from baseline to the event peak), duration (measured at half-maximal amplitude of the event), risetime (measured from the event onset to the event peak), and latency (measured from stimulus artifact to the event onset). An index of short-term synaptic plasticity was calculated as the ratio of the amplitudes of the first and last EJP within each individual stimulus train. Summation of EJPs was corrected for by measuring the amplitude of the final EJP from the peak to an extrapolation of the decay phase of the previous EJP. The results for each parameter were averaged into 5°C bins, such that an individual animal contributed one value only per temperature bin in statistical comparisons. The datasets consisted of recordings from 10 control and nine heat shock animals at 1.0 mM [Ca^{2+}], 11 control and 12 heat shock animals at 2.0 mM [Ca^{2+}], and seven control and eight heat shock animals at 3.0 mM [Ca^{2+}]. Significance ($p < .05$) was assessed with a Mann-Whitney rank sum test, t test, or two-way analysis of variance (ANOVA) with the appropriate posthoc test (Tukey test) where applicable. For statistical assessment where conditions of normality and equal variance were not met, the data have been appropriately transformed. All values are reported as mean ±S.E.M.

RESULTS

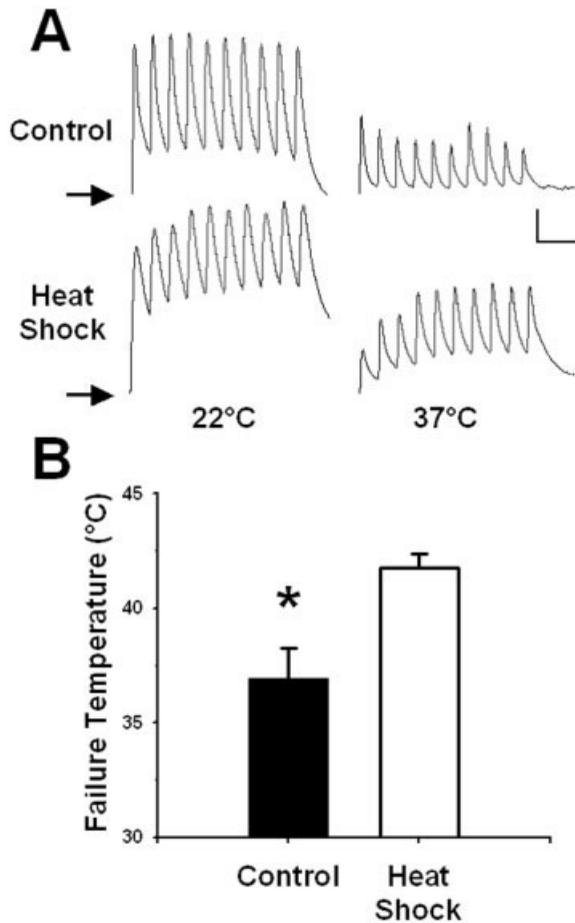### Heat Shock Increases Intrinsic Membrane Properties

Any effect of heat shock on short-term plasticity (Barclay and Robertson, 2001) could be a consequence of an alteration to the electrophysiological properties of the postsynaptic membrane. It is thus necessary to determine whether heat shock affects the intrinsic properties of the membrane. Prior exposure to heat shock increased both the input resistance [Fig. 1(A)] and time constant of the postsynaptic membrane [Fig. 1(B)]. Input resistance (two-way ANOVA, $F = 5.92$, $df = 1$, $p = .02$) and time constant of decay (two-way ANOVA, $F = 12.23$, $df = 3$, $p < .01$) were significantly higher in heat shock preparations. Although both parameters were reduced with increasing temperature ($p < .01$), their thermosensitivities were unaffected by heat shock.
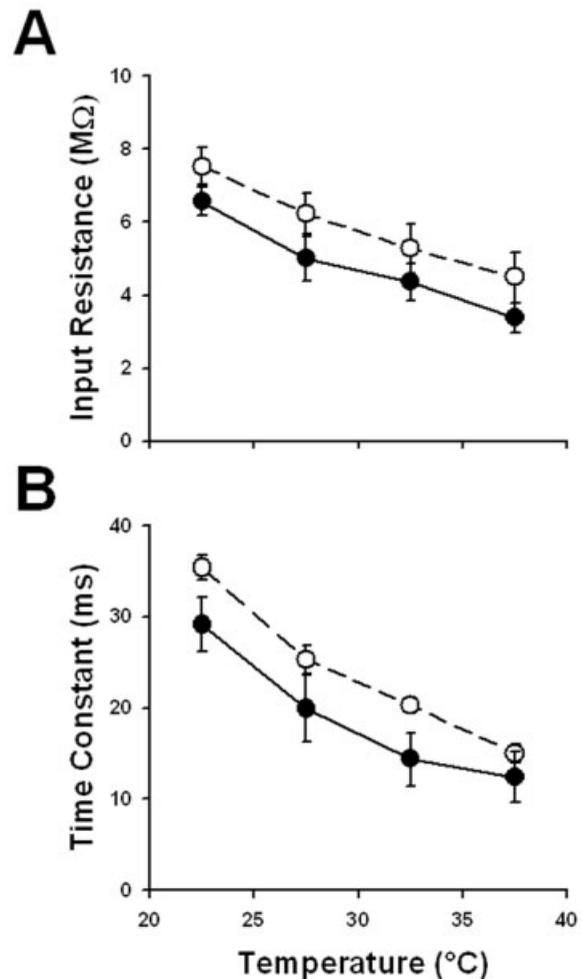
Figure 1 Heat shock modifies the passive electrical properties of the larval *Drosophila* postsynaptic membrane. (A) Absolute values of membrane input resistance were increased at all temperatures in heat shock preparations (open circles) in comparison to controls (closed circles). (B) Absolute values of membrane time constant were also increased at all temperatures in heat shock preparations. There are individual significant differences between temperature bins in the 20–25°C range ($p = .01$) and the 30–35°C range ($p = .04$). The thermosensitivities of both input resistance and time constant were not affected by prior heat shock exposure. Values are means ± S.E.

Thermoprotection of the Synapse Induced by Heat Shock at 1.0 mM [Ca^{2+}]

Trains of EJPs were recorded at 1.0 mM [Ca^{2+}] in larval abdominal muscle 6, segment 3 continuously from room temperature until synaptic failure. Characteristic expected changes in EJP parameters were observed in both control and heat shock preparations with an increase in temperature [Fig. 2(A)]; temporal
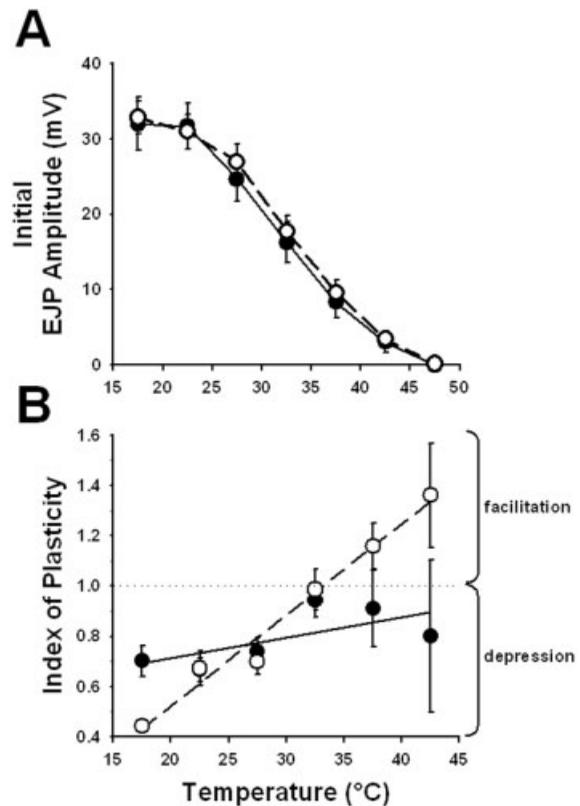
parameters of individual EJPs [Fig. 3(A) and data not shown; see also Discussion].

Heat Shock Affects Synaptic Plasticity at the *Drosophila* Neuromuscular Junction

Heat shock has previously been shown to enhance short-term synaptic plasticity and increase its temperature sensitivity at a facilitating synapse (Barclay and Robertson, 2001). During high-frequency stimulation,

Figure 2 Induction of synaptic thermoprotection in *Drosophila* by prior heat shock. (A) Representative traces of excitatory junctional potentials (EJPs) recorded from larval abdominal muscle fiber 6, segment 3 in control and heat shock preparations. Both control and heat shock preparations exhibited synaptic depression in response to 40 Hz tetanic stimulation. The timing and amplitude of EJPs were decreased with increased temperature. Note that short-term synaptic plasticity increased with temperature. Scale bars represent 10 mV and 50 ms. Arrows indicate the resting membrane potential prior to stimulation. (B) The upper temperature limit for synaptic transmission was increased by 5°C in heat shock preparations. Values are means ±S.E.

parameters (latency, risetime, duration) and amplitude were all reduced with increasing temperature ($p < .01$). In heat shock preparations, the temperature at which synaptic transmission failed was significantly increased by 4.8°C [Fig. 2(B); $p < .01$] and the variability in the upper temperature limit was greatly reduced [see e.g., error bars in Fig. 2(B); $p < .01$]. In contrast to previous recorded effects of heat shock on excitatory junctional currents at individual synaptic boutons of the *Drosophila* larvae (Karunanithi et al., 1999), heat shock surprisingly did not alter specific

Figure 3 Alteration of short-term synaptic plasticity in heat shock preparations. (A) During high-frequency stimulation, the amplitudes of the initial EJPs are identical in control (closed circles) and heat shock (open circles) preparations at all temperatures. (B) The extent of synaptic depression at room temperature at the larval neuromuscular junction is enhanced in heat shock preparations in comparison with controls. As well, the thermosensitivity of synaptic plasticity is increased in heat shock preparations. Synaptic plasticity was found to be thermosensitive in heat shock preparations only. There are individual significant differences between temperature bins in the 15–20°C range ($p < .01$) and the 40–45°C range ($p = .03$). Index of plasticity was calculated as the ratio of the amplitudes of the first and last EJP within each stimulus train. Values are means ±S.E.

however, the *Drosophila* neuromuscular junction exhibits depression in both control and heat shock preparations recorded at room temperature in 1 mM [Ca^{2+}] [Fig. 2(A)]. Although there was no difference in the amplitude of the first EJP in the train at any temperature [Fig. 3(A)], both the thermosensitivity of synaptic plasticity and the endogenous extent of synaptic depression were affected by prior exposure to heat shock [Fig. 3(B)]. As temperature increased, control preparations continued to exhibit synaptic depression. In contrast, the index of plasticity in heat shock preparations increased with temperature such that, at elevated temperatures, they displayed facilitation. Although no significant differences in plasticity between control and heat shock preparations occurred (two-way ANOVA, $F = 0.03$, $df = 1$, $p = .87$), there was a significant effect of temperature ($F = 8.25$, $df = 3$, $p < .01$). The effect of temperature was found to be dependent on heat shock, as there was a statistically significant interaction between the two variables ($F = 3.24$, $df = 3$, $p = .01$). Closer statistical examination revealed that the effect of temperature was significant for only heat shock preparations. Synaptic plasticity was unaffected by temperature in controls (Tukey test, $p > .05$), but was temperature sensitive in heat shock preparations (Tukey test, $p < .01$). Furthermore, at room temperature, the endogenous extent of synaptic depression was significantly greater in heat shock preparations in comparison to controls (Tukey Test, $p < .01$). The effect of heat shock on the thermosensitivity of synaptic plasticity was independently confirmed by statistical analysis of linear regressions. Plasticity in control preparations was insensitive to temperature ($r^2 = 0.46$, $p = .14$) with a linear regression slope of $0.008 \pm 0.004°C^{-1}$, whereas there was a significant linear relationship with temperature for heat shock preparations ($r^2 = 0.98$, $p < .01$), with a slope of $0.036 \pm 0.003°C^{-1}$. Heat shock significantly increased the slope of the linear relationship between plasticity and temperature (analysis of covariance, $t = 5.6$, $df = 15$, $p < .01$; Sokal and Rohlf, 1981).

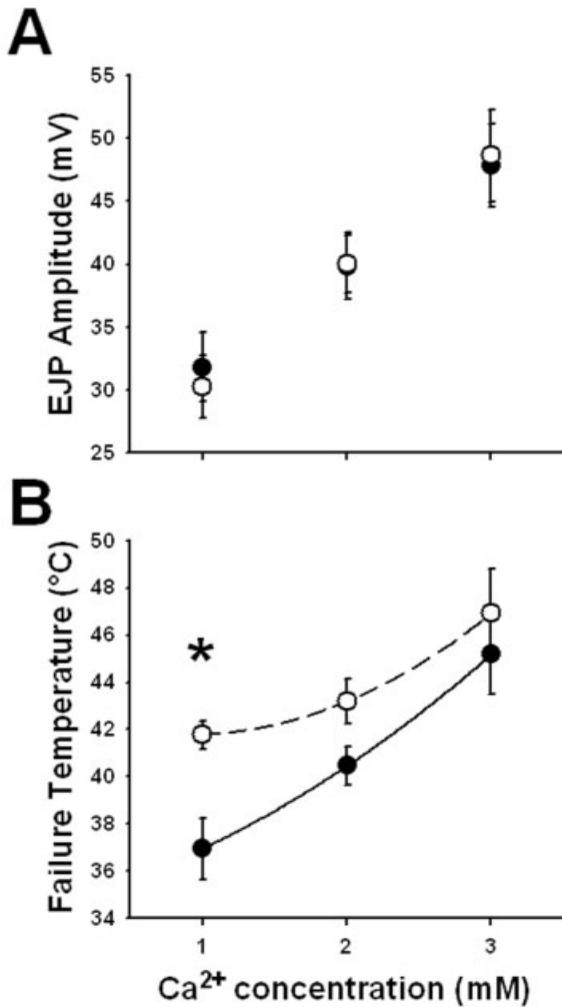## Increasing Extracellular [Ca^{2+}] Nullifies the Thermoprotective Effect of Heat Shock

A mechanism for short-term synaptic depression involves a depletion of the readily releasable pool of vesicles, which is known to be influenced by levels of presynaptic calcium (Wang and Kaczmarek, 1998; Dittman et al., 2000; Zucker and Regehr, 2002). To investigate this hypothesis further, the amount of calcium influx at the neuromuscular junction was manip-

ulated by varying the level of extracellular calcium. EJPs were recorded, in control and heat shock preparations, continuously from room temperature until synaptic failure at 1, 2, and 3 mM extracellular [Ca^{2+}]. Due to the effects of calcium on quantal content (Augustine and Charlton, 1986), at room temperature EJP amplitude increased with increasing extracellular calcium [Fig. 4(A); two-way ANOVA, $F = 15.573$, $df = 2$, $p < .01$]. The effect of calcium on EJP amplitude was further confirmed by a posthoc Tukey test (1 mM vs. 2 mM [Ca^{2+}], $p < .01$; 1 mM vs. 3 mM [Ca^{2+}], $p < .01$; 2 mM vs. 3 mM [Ca^{2+}], $p = .04$). This was expected, as it has been reported that the strength of synaptic transmission is sensitive to extracellular calcium concentration up to 4 mM [Ca^{2+}] (Dawson-Scully et al., 2000). EJP amplitude was not different in heat shock preparations in comparison with controls. All measured EJP parameters were decreased with increasing temperature, regardless of calcium concentration [see, e.g., Fig. 2(A)]. The upper temperature limit for synaptic transmission was increased by raising the calcium concentration in the extracellular solution [Fig. 4(B)]. In control preparations, failure occurred at 37.0°C at 1.0 mM [Ca^{2+}], 40.5°C at 2.0 mM [Ca^{2+}], and 45.2°C at 3.0 mM [Ca^{2+}]; this calcium-induced increase was significant ($p < .01$). Heat shock also increased the failure temperature for synaptic transmission ($p < .01$); however, this thermoprotective effect of heat shock was only evident at lower levels of extracellular calcium. Whereas at 1.0 mM [Ca^{2+}] prior exposure to heat shock significantly increased the upper temperature limit of synaptic transmission ($p < .01$), as extracellular calcium concentration was elevated this difference between control and heat shock preparations was abolished. Furthermore, at 1.0 mM [Ca^{2+}], the failure temperatures of control preparations were significantly more variable than in heat shock preparations [see, e.g., error bars in Fig. 4(B); $p < .01$] and this difference in variability was also abolished by the increase in extracellular calcium.

Heat Shock Induced Thermoprotection Is Mimicked by Increasing Extracellular Calcium

Calcium concentration altered individual EJP parameters in control preparations (Figs. 5 and 6), mimicking previously published results on the effects of heat shock (Dawson-Scully and Robertson, 1998; Karunanithi et al., 1999; Barclay and Robertson, 2000, 2001). The measured latency of synaptic transmission was significantly reduced by raising extracellular cal-
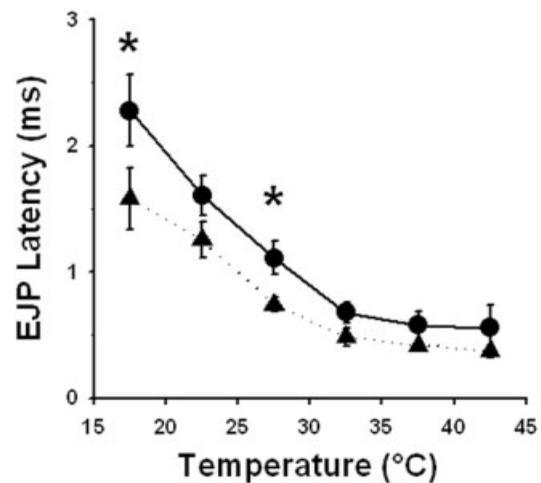
Figure 4 Increasing extracellular calcium nullified the thermoprotective effect of heat shock at *Drosophila* synapses. (A) At room temperature, EJP amplitude is significantly increased by increasing extracellular calcium concentration. Amplitudes at all three concentrations are significantly different. (B) The upper temperature limit for synaptic transmission in control preparations was increased by increasing the calcium concentration in the extracellular media, mimicking the effect of prior heat shock. Open circles represent heat shock preparations, closed circles represent control preparations. An increase in the upper temperature limit for successful synaptic transmission by heat shock occurred only at 1.0 mM [Ca^{2+}] ($p < .01$). Values are means ±S.E.

cium (two-way ANOVA, $F = 12.56$, $df = 2$, $p < .01$; Fig. 5).

Due to the effects of calcium concentration on quantal content (Augustine and Charlton, 1986), the amplitude of EJPs was significantly increased with extracellular calcium over the temperature range investigated [Fig. 6(A); two-way ANOVA, $F = 20.72$,

$df = 2$, $p < .01$]. The effect of calcium was confirmed by a posthoc Tukey test (1 mM vs. 2 mM [Ca^{2+}], $p < .01$; 1 mM vs. 3 mM [Ca^{2+}], $p < .01$; 2 mM vs. 3 mM [Ca^{2+}], $p = .03$). To compensate for this effect of calcium concentration, the remaining EJP parameters were normalized with their value at room temperature in order to make unbiased comparisons of the thermosensitivity (i.e., the effect of temperature on an EJP parameter) of synaptic transmission across calcium levels. All EJP parameters were significantly decreased by temperature at all calcium concentrations (two-way ANOVAs, $p < .01$). The thermosensitivities of both EJP amplitude (two-way ANOVA, $F = 11.07$, $df = 2$, $p < .01$) and duration (two-way ANOVA, $F = 10.06$, $df = 2$, $p < .01$) were reduced with the elevation of extracellular calcium [Fig. 6(B,C)]. Not all parameters of the EJP were affected; normalized EJP risetime was unaffected by extracellular calcium [Fig. 6(D); $p > .05$].

Increasing extracellular calcium also significantly altered short-term synaptic plasticity in controls (Fig. 7; two-way ANOVA, $F = 4.28$, $df = 2$, $p = .02$) in a similar manner to heat shock preparations (see, e.g., Fig. 4), although there was no significant interaction between calcium and temperature ($F = 1.06$, $df = 10$, $p = .40$). An effect of calcium to increase the thermosensitivity of plasticity, however, was confirmed with linear regression analysis. Plasticity in controls

Figure 5 Increased extracellular calcium reduced EJP latency in control preparations. Latencies were significantly decreased at 3.0 mM [Ca^{2+}] (triangles) in comparison to 1.0 mM [Ca^{2+}] preparations (circles). There are individual significant differences between temperature bins in the 15–20°C range ($p < .01$) and the 25–30°C range ($p = .02$). In order to enhance the visual impact of the effect of extracellular calcium concentration, in this and subsequent figures, only the results for 1.0 and 3.0 mM [Ca^{2+}] are presented. Values are means ±S.E.
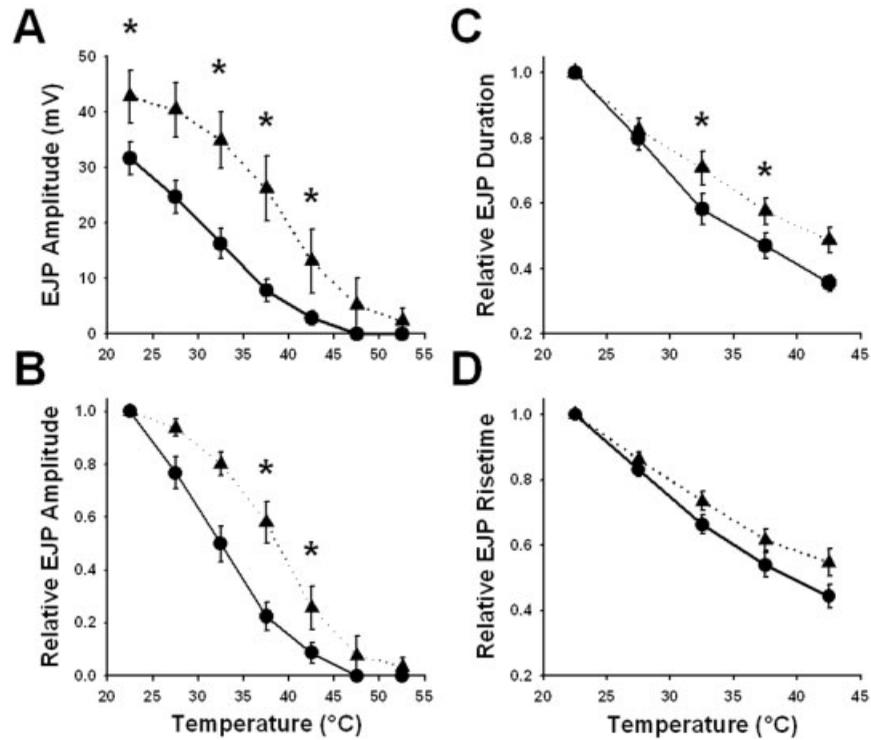
Figure 6 Induction of thermoprotection of EJP amplitude and duration in control preparations by increasing extracellular calcium concentration. (A) Absolute EJP amplitude was increased in 3.0 mM [Ca^{2+}] preparations (triangles) in comparison with preparations in 1.0 mM [Ca^{2+}] (circles). There are individual significant differences between temperature bins in the 20–25°C range (p = .04) and the 30–35°C, 35–40°C, and 40–45°C ranges (p < .01). Due to the effects of calcium concentration on quantal content, the remaining EJP parameters were normalized to their value at room temperature. (B) The thermosensitivity of normalized EJP amplitude was significantly reduced in 3.0 mM [Ca^{2+}] preparations compared with 1.0 mM [Ca^{2+}]. There are individual significant differences between temperature bins in the 35–40°C and 40–45°C ranges (p < .01). (C) The thermosensitivity of normalized EJP duration was significantly reduced in 3.0 mM [Ca^{2+}] preparations in comparison with 1.0 mM [Ca^{2+}]. There are individual significant differences between temperature bins in the 30–35°C range (p < .05) and the 35–40°C range (p < .01). (D) The thermosensitivity of normalized EJP risetime was unaffected by extracellular calcium concentration. Values are means ±S.E.

at 1.0 mM [Ca^{2+}] was insensitive to temperature (r^2 = 0.46, p = .14), with a slope of 0.008 ± 0.004°C^{-1}. At 3.0 mM [Ca^{2+}] temperature increased the index of plasticity (r^2 = 0.8, p = .02), with a slope of 0.013 ± 0.003°C^{-1}. Although temperature had a significant effect on plasticity when calcium concentration was elevated, the regression slope was unaffected (analysis of covariance, t = 1, df = 13, p > .05).

DISCUSSION

In this article we confirm and extend previous results by demonstrating that prior heat shock imparts synaptic thermoprotection by increasing the upper temperature limit for evoked exocytosis, concomitant with an enhancement of endogenous short-term synaptic plasticity and an increase in its thermosensitivity. We show further that increasing extracellular calcium levels negated the thermoprotective effects of heat shock by preventing the heat shock-mediated increase in the upper temperature limit for synaptic transmission. Finally, we demonstrate that elevation of the extracellular calcium concentration itself could induce synaptic thermoprotection, thus mimicking an established effect of heat shock at the synapse. These data support the hypothesis that heat shock acts presynaptically via an effect on residual calcium-dependent mechanisms within the nerve terminal.
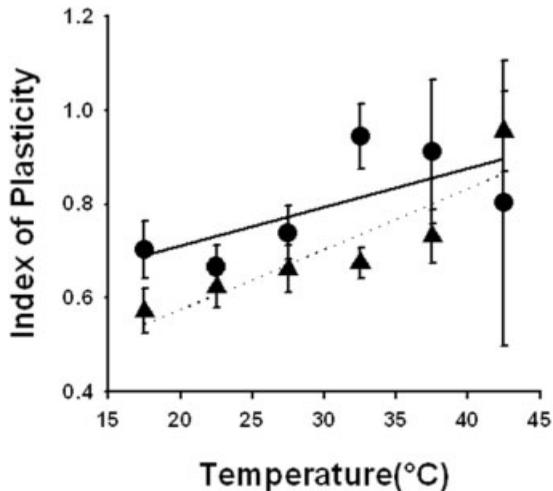
Figure 7 Short-term synaptic plasticity is altered by increasing extracellular calcium in control preparations. Similar to the effect of heat shock at 1.0 mM [Ca^{2+}], the thermosensitivity of synaptic plasticity is increased in 3.0 mM [Ca^{2+}] preparations (triangles) in comparison with 1.0 mM [Ca^{2+}] (circles). There are individual significant differences between temperature bins in the 30–35°C range (p = .04). Values are means ±S.E.

Changes to Passive Membrane Properties

Prior exposure to heat shock increased both the membrane input resistance and time constant of decay, without affecting their thermosensitivities (Fig. 1). For each parameter, there was a rightward shift of 5–6°C in the graphical representation of their temperature relationship. Interestingly, this shift approximates the 5° increase in the upper temperature threshold for synaptic transmission in heat shock larvae. It is most likely that the effects on the passive membrane properties are due to heat shock-modification to the temperature sensitivity of the postsynaptic leak current. Do the effects of heat shock to the passive properties of the membrane, however, account for the subsequent physiological effects on synaptic plasticity?

If the increase in input resistance in heat shock preparations is physiologically meaningful as to have an effect on membrane depolarization, then EJP amplitude should be increased for heat shock larvae. No difference in amplitude, however, was found between control and heat shock preparations. Furthermore, the increase in input resistance is not being compensated for by an equivalent decrease in current, as the opposite has been previously shown (Karunanithi et al., 1999). With similar reasoning, the increase in membrane time constant would imply an effect on EJP

duration. This is also not evident in heat shock preparations. Therefore, we conclude that although the passive membrane properties are increased by prior heat shock, it is unlikely that this change is contributing to the effects on synaptic plasticity.

Absence of Effect of Heat Shock on Individual EJP Parameters

The effect of heat shock on EJCs at individual 1b-type boutons of larval *Drosophila* is to decrease the thermosensitivity of synaptic strength (Karunanithi et al., 1999). We report here, however, that there is no effect of prior heat shock on the amplitude of integrated EJPs reflecting the summed response of the muscle fiber's boutons [Fig. 3(A)]. A lack of thermoprotection may simply be due to the significant effect at single boutons being masked by variation between all boutons contained in the muscle fiber. There is, however, an alternative, more likely explanation.

In the larva, ventral abdominal muscle 6, segment 3 is innervated by two motor axons contained within the motor nerve: a "fast" axon, generating large EJPs, and a "slow" axon, generating smaller EJPs (Kurdyak et al., 1994), which are physiologically reminiscent of the two excitatory motor axons in other invertebrate preparations, such as the locust hindleg (Burrows, 1996) and crustacean leg (Atwood, 1976). Previous research on the locust hindleg has shown that heat shock has differential effects at the fast and slow extensor tibial neuromuscular junctions (Barclay and Robertson, 2000, 2001). The thermosensitivity of synaptic strength at the fast neuromuscular junction is reduced by heat shock, whereas the absolute strength at room temperature is unaffected. In contrast, heat shock reduces the absolute synaptic strength at the slow neuromuscular junction, but does not affect its thermosensitivity. The *Drosophila* preparation is limited in that it is prohibitively difficult to separate physiologically synaptic transmission of the two motor axons and assay muscle fiber EJPs at variable temperatures, as stimulus threshold differentials for the two axons established at room temperature (see, e.g., Lnenicka and Keshishian, 2000) disappear as the temperature is raised (J. W. Barclay, unpublished observations). We would argue that different effects of heat shock at "fast" and "slow" neuromuscular junctions, if they were to exist also in the two motor axon types in *Drosophila*, would offset each other, and thus, mask any effect on integrated EJP amplitude.

Short-Term Synaptic Plasticity and Modification of Residual Calcium

Prior exposure to heat shock was found to alter short-term synaptic plasticity in two ways. At the neuromuscular junction in larval *Drosophila*, both the endogenous extent of synaptic depression and its thermosensitivity were increased by heat shock [Fig. 3(B)]. A similar response has been previously reported at the locust hindleg neuromuscular junction (Barclay and Robertson, 2001), albeit for short-term facilitation. While there are intrinsic differences between the two insect preparations, it is intriguing that heat shock imparted the same effect regardless of the endogenous direction of synaptic plasticity (i.e., facilitation or depression). A tempting hypothesis is that a primary neurophysiological response to a stressful environment is to enhance the short-term ability to regulate the strength of synaptic connections.

Enhancement of synaptic facilitation would indicate an effect on residual presynaptic calcium levels during repetitive synaptic transmission (Katz and Miledi, 1968; Kamiya and Zucker, 1994); however, transmission at the larval synapse depresses at room temperature. Although there is some evidence implicating the involvement of receptor desensitization at the *Drosophila* neuromuscular junction during repetitive stimulation (Adelsberger et al., 1997), short-term synaptic depression is believed to be largely attributed to the rapid depletion of the readily releasable pool of vesicles, which is influenced by presynaptic calcium levels (Wang and Kaczmarek, 1998; Dittman et al., 2000; Zucker and Regehr, 2002). Thus, a simple model for all forms of short-term modification of synaptic strength involves presynaptic residual calcium (Zucker, 1999; Dittman et al., 2000; Zucker and Regehr, 2002). Our results would support such a model as a common mechanism for short-term synaptic plasticity, demonstrated by the transition between depression and facilitation at the same neuromuscular junction during exposure to a monotonic increase in temperature in heat shock *Drosophila* preparations. Although the extent of depression can be affected by the initial release probability, this is unlikely to be a factor here as initial EJP amplitudes were identical in control and heat shock preparations at all temperatures [Fig. 3(A)]. Therefore, the most parsimonious conclusion is that the difference in synaptic depression is a result of heat shock affecting either residual calcium levels or the presynaptic targets/effectors of residual calcium during short-term changes in synaptic strength. This conclusion would agree with the previous work demonstrating an effect of heat shock on residual calcium at a facilitating synapse in the locust (Barclay and Robertson, 2001).

Raising the concentration of extracellular calcium imparts thermoprotection to synaptic transmission in control preparations of *Drosophila* larvae, mimicking the effect of heat shock [Fig. 4(B)]. Furthermore, it also induced a thermoprotection of EJP amplitude and duration [Fig. 6(B,C)]. These results duplicate established neuroprotective effects of heat shock evident at individual larval *Drosophila* boutons (Karunanithi et al., 1999) and at locust synapses (Dawson-Scully and Robertson, 1998; Barclay and Robertson, 2000, 2001). Experimentally increasing calcium levels external to the nerve terminal would increase the driving force on calcium, demonstrated by the increase in the absolute strength of synaptic transmission with increased calcium [Figs. 4(A) and 6(A)]. Thus, during a presynaptic depolarization, just prior to vesicular release, there would be an increased amount of calcium entering the nerve terminal. At elevated temperatures this increase in calcium influx could offset the faster closing kinetics of the voltage-dependent calcium channel at the nerve terminal or the increased rate of calcium removal from the active zone (Sabatini and Regehr, 1999). It is unlikely that elevated calcium would prevent a heat-induced failure of action potential conduction, as the extracellular application of divalent cations such as calcium decreases axonal excitability (Frankenhaeuser and Hodgkin, 1957). As increased extracellular calcium negates the thermoprotective effect of heat shock at the synapse and itself imparts thermoprotection of synaptic transmission, it is likely that heat shock is acting through an effect on presynaptic calcium at the nerve terminal.

Our results do not specify the physiological mechanism upstream of the heat shock effect on residual presynaptic calcium. It could be due to altered calcium entry, via a heat shock effect on action potential breadth (Jackson et al., 1991; Byrne and Kandel, 1996; Sugita et al., 1997) as heat shock has been demonstrated to alter K^+ channel kinetics, and consequently action potential duration (Ramirez et al., 1999; Wu et al., 2001). Alternatively, heat shock could alter presynaptic calcium dynamics via a mechanism independent of spike broadening (Chen and Regehr, 1997). Further experiments will be required to determine whether heat shock preserves calcium levels by affecting calcium entry and/or its removal.

Potential Molecular Mechanisms

An obvious putative mechanism for the effects of heat shock is through inducible chaperones, such as the heat shock proteins (Parsell and Lindquist, 1993). We

have previously shown that the onset and duration for heat shock-induced thermoprotection parallel those of the timeframe for up-regulated hsp70 protein expression (Karunanithi et al., 1999), and the extent of thermoprotection is enhanced in genetically manipulated *Drosophila* carrying elevated copies of the hsp70 gene (Karunanithi et al., 2002). The results presented in this study give experimental support to an association between residual presynaptic calcium and heat shock-induced thermoprotection. The chaperone function of heat shock proteins requires ATP hydrolysis, which is known to regulated by calcium (Sriram et al., 1997) and exposure to bimoclomol, a chemical inducer of hsp70 that has been shown to affect calcium handling in mammalian ventricular myocardium (Nánási et al., 2000). As a cellular chaperone, hsp70 could act to stabilize proteins intrinsic to the exocytotic machinery from stress-induced denaturation. The ATPase activity of heat shock proteins, and thus their chaperone ability, require activation by members of the DnaJ family of proteins (Kelley, 1998). At the presynaptic nerve terminal, CSP, a member of this family, can activate the ATPase activity of hsp70 (Chamberlain and Burgoyne, 1997) and has chaperone abilities of its own (Chamberlain and Burgoyne, 2000). Recently it has been shown that CSP exists as a trimeric chaperone complex with Hsc70, the heat shock protein cognate, and the SGT protein (Tobaben et al., 2001). It is worth noting that, similar to the results presented here, *Drosophila* larvae with null mutations in the *csp* or *hsc70* gene have temperature-sensitive impairments of neurotransmitter release (Zinsmaier et al., 1994; Bronk et al., 2001) and that either mutational phenotype can be restored by elevating levels of extracellular calcium (Dawson-Scully et al., 2000; Bronk et al., 2001). One cannot rule out, however, an effect of heat shock through alternative mechanisms, as environmental stress is known to up-regulate a host of different proteins with diverse consequences (Leemans et al., 2000; Zou et al., 2000).

In conclusion, exposure to a prior heat stress alters short-term depression of the *Drosophila* synapse and its thermosensitivity. This is potentially a neurophysiological response to increase the plasticity of the synapse, thereby increasing its homeostatic potential in the face of increased variability of the external environment. Furthermore, these results support the idea that heat shock acts mechanistically through an alteration to the physiological actions of residual calcium in the presynaptic nerve terminal. Future work, however, is required to determine whether heat shock affects calcium entry and/or removal from the active zone and to characterize any accessory proteins critical for the mechanism of presynaptic alteration.

REFERENCES

Adelsberger H, Heckmann M, Dudel J. 1997. The amplitude of quantal currents is reduced during short-term depression at neuromuscular synapses in Drosophila. Neurosci Lett 225:5–8.

Atwood HL. 1976. Organization and synaptic physiology of crustacean neuromuscular systems. Prog Neurobiol 7:291–391.

Atwood HL, Govind CK, Wu C-F. 1993. Differential ultrastructure of synaptic terminals on ventral longitudinal abdominal muscles in Drosophila larvae. J Neurobiol 24:1008–1024.

Augustine GJ, Charlton MP. 1986. Calcium dependence of presynaptic calcium current and postsynaptic response at the squid giant synapse. J Physiol (Lond) 381:619–640.

Barclay JW, Atwood HL, Robertson RM. 2002. Impairment of central pattern generation in Drosophila cysteine string protein mutants. J Comp Physiol A 188:71–78.

Barclay JW, Robertson RM. 2000. Heat shock-induced thermoprotection of hindleg motor control in the locust. J Exp Biol 203:941–950.

Barclay JW, Robertson RM. 2001. Enhancement of short-term synaptic plasticity by prior environmental stress. J Neurophysiol 85:1332–1335.

Bronk P, Wenniger JJ, Dawson-Scully K, Guo X, Hong S, Atwood HL, Zinsmaier KE. 2001. Drosophila Hsc70-4 is critical for neurotransmitter exocytosis in vivo. Neuron 30:475–488.

Burrows M. 1996. The neurobiology of an insect brain. New York: Oxford University Press. 682 p.

Byrne JH, Kandel ER. 1996. Presynaptic facilitation revisited: state and time dependence. J Neurosci 16:425–435.

Chamberlain LH, Burgoyne RD. 1997. Activation of the ATPase activity of heat-shock proteins Hsc70/Hsp70 by cysteine-string protein. Biochem J 322:853–858.

Chamberlain LH, Burgoyne RD. 2000. Cysteine-string protein: the chaperone at the synapse. J Neurochem 74:1781–1790.

Chen C, Regehr WG. 1997. The mechanism of cAMP-mediated enhancement at a cerebellar synapse. J Neurosci 17:8687–8694.

Craig EA, Weissman JS, Horwich AL. 1994. Heat shock proteins and molecular chaperones: mediators of protein conformation and turnover in the cell. Cell 78:365–372.

Dawson-Scully K, Bronk P, Atwood HL, Zinsmaier KE. 2000. Cysteine-string protein increases the calcium sen-

sitivity of neurotransmitter exocytosis in Drosophila. J Neurosci 20:6039–6047.

Dawson-Scully K, Robertson RM. 1998. Heat shock protects synaptic transmission in flight motor circuitry of locusts. NeuroReport 9:2589–2593.

Dittman JS, Kreitzer AC, Regehr WG. 2000. Interplay between facilitation, depression, and residual calcium at three presynaptic terminals. J Neurosci 20:1374–1385.

Estruch F. 2000. Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast. FEMS Microbiol Rev 24:469–486.

Fink AL. 1999. Chaperone-mediated protein folding. Physiol Rev 79:425–449.

Fink SL, Chang LK, Ho DY, Sapolsky RM. 1997. Defective herpes simplex virus vectors expressing the rat brain stress-inducible heat shock protein 72 protect cultured neurons from severe heat shock. J Neurochem 68:961–969.

Fisher SA, Fischer TM, Carew TJ. 1997. Multiple overlapping processes underlying short-term synaptic enhancement. Trends Neurosci 20:170–177.

Frankenhaeuser B, Hodgkin AL. 1957. The action of calcium on the electrical properties of squid axons. J Physiol (Lond) 137:218–244.

Hartl FP, Hayer-Hartl M. 2002. Molecular chaperones in the cytosol: from nascent chain to folded protein. Science 295:1852–1858.

Jackson MB, Konnerth A, Augustine GJ. 1991. Action potential broadening and frequency-dependent facilitation of calcium signals in pituitary nerve terminals. Proc Natl Acad Sci USA 88:380–384.

Jolly C, Morimoto RI. 2000. Role of the heat shock response and molecular chaperones in oncogenesis and cell death. J Natl Cancer Inst 92:1564–1572.

Kamiya H, Zucker RS. 1994. Residual Ca^{2+} and short-term synaptic plasticity. Nature 371:603–606.

Karunanithi S, Barclay JW, Brown IR, Robertson RM, Atwood HL. 2002. Enhancement of presynaptic performance in transgenic Drosophila overexpressing heat shock protein HSP70. Synapse 44:8–14.

Karunanithi S, Barclay JW, Robertson RM, Brown IR, Atwood HL. 1999. Neuroprotection at Drosophila synapses conferred by prior heat shock. J Neurosci 19:4360–4369.

Katz B, Miledi R. 1968. The role of calcium in neuromuscular facilitation. J Physiol (Lond) 195:481–492.

Kelley WL. 1998. The J-domain family and the recruitment of chaperone power. Trends Biochem Sci 23:222–227.

Kelty JD, Noseworthy PA, Feder ME, Robertson RM, Ramirez J-M. 2002. Thermal preconditioning and heat-shock protein 72 preserve synaptic transmission during thermal stress. J Neurosci 22:RC193 (1–6).

Kiang JG, Ding XZ, McClain DE. 1998. Overexpression of hsp-70 attenuates increases in $[Ca^{2+}]_i$ and protects human epidermoid A-431 cells after chemical hypoxia. Toxicol Appl Pharmacol 149:185–194.

Kurdyak P, Atwood HL, Stewart BA, Wu C-F. 1994. Differential physiology and morphology of motor axons to ventral longitudinal muscles in larval Drosophila. J Comp Neurol 350:463–472.

Leemans R, Egger B, Loop T, Kammermeier L, He H, Hartmann B, Certa U, Hirth F, Reichert H. 2000. Quantitative transcript imaging in normal and heat-shocked Drosophila embryos by using high-density oligonucleotide arrays. Proc Natl Acad Sci USA 97:12138–12143.

Lnenicka GA, Keshishian H. 2000. Identified motor terminals in Drosophila larvae show distinct differences in morphology and physiology. J Neurobiol 43:186–197.

Mailhos C, Howard MK, Latchman DS. 1993. Heat shock protects neuronal cells from programmed cell death. Neurosci 55:621–627.

Nánási PP, Sárközi S, Szigeti G, Jóna I, Szegedi C, Szabó Á, Bányász T, Magyar J, Szigligeti P, Körtvély Á, Csernoch L, Kovács L, Jednákovits A. 2000. Biphasic effect of bimoclomol on calcium handling in mammalian ventricular myocardium. Brit J Pharmacol 129:1405–1412.

Ohtsuka K, Suzuki T. 2000. Roles of molecular chaperones in the nervous system. Brain Res Bull 53:141–146.

Parsell DA, Lindquist S. 1993. The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. Annu Rev Genet 27:437–496.

Sabatini BL, Regehr WG. 1999. Timing of synaptic transmission. Annu Rev Physiol 61:521–542.

Smith PJS, Hammar K, Tytell M. 1995. Effects of exogenous heat shock protein (hsp70) on neuronal calcium flux. Biol Bull 189:209–210.

Sokal RR, Rohlf FJ. 1981. Biometry. 2nd Ed. New York: W.H. Freeman & Company. 859 p.

Sriram M, Osipiuk J, Freeman BC, Morimoto RI, Joachimiak A. 1997. Human hsp70 molecular chaperone binds two calcium ions within the ATPase domain. Structure 5:403–414.

Stewart BA, Atwood HL, Renger JJ, Wang J, Wu C-F. 1994. Improved stability of Drosophila larval neuromuscular preparations in haemolymph-like physiological solutions. J Comp Physiol A 175:179–191.

Sugita S, Baxter DA, Byrne JH. 1997. Differential effects of 4-aminopyridine, serotonin, and phorbol esters on facilitation of sensorimotor connections in Aplysia. J Neurophysiol 77:177–185.

Tobaben S, Thakur P, Fernández-Chacón R, Südhof TC, Rettig J, Stahl B. 2001. A trimeric protein complex functions as a synaptic chaperone machine. Neuron 31:987–999.

Torrecilla I, Leganés F, Bonilla I, Fernández-Piñas F. 2000. Use of recombinant aequorin to study calcium homeostasis and monitor calcium transients in response to heat and cold shock in cyanobacteria. Plant Physiol 123:161–175.

Wang L-Y, Kaczmarek LK. 1998. High-frequency firing helps replenish the readily releasable pool of synaptic vesicles. Nature 394:384–388.

Wu BS, Walker VK, Robertson RM. 2001. Heat shock-

induced thermoprotection of action potentials in the locust flight system. J Neurobiol 49:188–199.

Yenari MA, Fink SL, Sun GH, Patel M, Kunis D, Onley D, Sapolsky RM, Steinberg GK. 1998. Gene therapy with HSP72 is neuroprotective in rat models of stroke and epilepsy. Ann Neurol 44:584–591.

Yenari MA, Giffard RG, Sapolsky RM, Steinberg GK. 1999. The neuroprotective potential of heat shock protein 70 (HSP70). Mol Med Today 5:525–531.

Zinsmaier KE, Eberle KK, Buchner E, Walter N, Benzer S. 1994. Paralysis and early death in cysteine string protein mutants of Drosophila. Science 263:977–980.

Zou S, Meadows S, Sharp L, Jan LY, Jan YN. 2000. Genome-wide study of aging and oxidative stress response in Drosophila melanogaster. Proc Natl Acad Sci USA 97:13726–13731.

Zucker RS. 1999. Calcium- and activity-dependent synaptic plasticity. Curr Opin Neurobiol 9:305–313.

Zucker RS, Regehr WG. 2002. Short-term synaptic plasticity. Annu Rev Phys 64:355–405.