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Enhancement of Presynaptic Performance in Transgenic *Drosophila* Overexpressing Heat Shock Protein HSP70

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ABSTRACT Prior heat shock confers protection to *Drosophila* synapses during subsequent heat stress by stabilizing quantal size and reducing the decline of quantal emission at individual synaptic boutons. The major heat shock protein Hsp70, which is strongly induced by high temperatures in *Drosophila,* may be responsible for this synaptic protection. To test this hypothesis, we investigated synaptic protection and stabilization at larval neuromuscular junctions of transgenic *Drosophila* which produce more than the normal amount of Hsp70 in response to heat shock. Overexpression of Hsp70 coincides with enhanced protection of presynaptic performance, assayed by measuring mean quantal content and percentage success of transmission. Quantal size was not selectively altered, indicating no effects of overexpression on postsynaptic performance. Thus, presynaptic mechanisms can be protected by manipulating levels of Hsp70, which would provide stability to neural circuits otherwise susceptible to heat stress. **Synapse 44:8–14, 2002.** © 2002 Wiley-Liss, Inc.

INTRODUCTION

Heat shock (brief exposure to sublethal temperatures) enables cells to survive subsequent exposure to high temperatures that would otherwise be lethal (Morimoto et al., 1994; Parsell and Lindquist, 1993). Synaptic transmission in the nervous system can also be sustained at high temperatures by prior heat shock. Several components of synaptic function are protected or stabilized (Dawson-Scully and Robertson, 1998; Karunanithi et al., 1999). Postsynaptic currents retain a more stable amplitude at high test temperatures (>31°C) compared to nonheat-shocked controls. Presynaptic performance is also improved: at high temperatures the percentage of failures of transmitter release is lower and the mean quantal output is higher in heat-shocked individuals (Karunanithi et al., 1999). These observations suggest rapid short-term adaptation of synaptic transmission to stressful environmental conditions.

The molecular mechanisms responsible for conferring neuroprotection following a heat shock have yet to be identified. In *Drosophila,* most protein synthesis is downregulated following a heat shock, but there is a striking induction of heat shock protein 70 (Hsp70). Expression of Hsp70 is not detectable in nonheat-shocked animals, but during heat shock it becomes the most prominent protein synthesized (Parsell and Lindquist, 1993). Upregulation of heat shock proteins, especially Hsp70, could be an important component of the protective response.

Since heat shock proteins preserve cellular integrity by preventing protein misfolding, aggregation, and denaturation during exposure to abnormally high temperatures (Parsell and Lindquist, 1993), their increased production could serve to stabilize and maintain synaptic transmission. A test of this hypothesis is available in *Drosophila,* since transgenic lines have been developed (Welte et al., 1995) which when heat-shocked produce higher than normal levels of Hsp70, due to insertion of 12 extra copies of the Hsp70 gene into the genome (making a total of 22 copies). In the present study, we compared synaptic performance

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in these transgenic larvae (*tra* line) with that in a control line (*cis* line) possessing the normal number of copies of the Hsp70 gene, but with the flanking P-element sequences at the same insertion sites, controlling for positional mutagenesis. The results of the test show that overexpression of Hsp70 alone is sufficient to improve presynaptic performance at high temperatures; however, postsynaptic performance is unaffected.

MATERIALS AND METHODS

Fly stocks

The *traII* and *cisII* lines of *Drosophila melanogaster* are extra copy and excision strains, respectively, with the sites of transgene insertion into chromosome II. The construction of these strains is described elsewhere (Welte et al., 1995). The extra copy strain, *traII,* contains 12 extra copies of the Hsp70 gene, an eye-color marker, w^{hs}, and flanking yeast recombination targets and P-elements. The excision strain, *cisII,* shares the same chromosomal sites of transgene insertion and flanking sequences but lacks the extra copies of the Hsp70 gene and eye-color marker. Fly stocks were reared at 25°C (60–70% relative humidity) on a standard cornmeal medium made with corn syrup.

Heat shock

Larvae were placed in a standard Petri dish containing filter paper moistened with phosphate buffer and taped shut to preserve humidity. They were heat-shocked at 36°C for 1 h, then allowed to recover at 25°C for 30 min (time of maximal Hsp70 protein induction) before electrophysiological experimentation. The same protocol was used to prepare larvae for Western blot analysis of heat-shocked third-instar larvae.

Western blot analysis of Hsp70

Nonheat-shocked control (NHS) and heat-shocked (HS) third-instar larvae from the *cis II* and *tra II* lines were collected, quick-frozen on dry ice, and stored at −70°C. Groups of five larvae were homogenized in 200 μl of 0.32 M sucrose in 1.5 ml microfuge tubes with 20 passes with a fitted Teflon pestle. Protein concentrations were determined using the Bio-Rad (Hercules, CA) protein assay. Aliquots of 25 μg of protein were solubilized by boiling for 5 min with an equal volume of dissociation buffer (8 M urea, 2% SDS, 2% β-mercaptoethanol, and 20% glycerol). PAGE was performed in the presence of SDS on 10% gels with a 5% stacking gel using the discontinuous buffer system of Laemmli (1970). The proteins were transferred onto nitrocellulose membranes for 16–18 h in a solution of 50 mM boric acid, 4 mM β-mercaptoethanol, and 2 mM EDTA, at 400 nA. Blots were stained with Ponceau S to check for equal loading of protein in all lanes.

For Western blot analysis of Hsp70 protein, the blots were washed for 10 min in 0.1 M PBS, pH 7.4, blocked for 2 h in 5% milk powder and PBS, and then incubated overnight in primary antibody diluted 1:10,000 (*Drosophila* Hsp70-specific monoclonal antibody 7FB; gift from Dr. S. Lindquist). Blots were then washed three times for 10 min each in PBS plus 0.1% Tween 20, incubated for 1 h in secondary antibody diluted 1:20,000 (rat IgG adsorbed with human IgG), and then washed three times for 5 min each in PBS plus 0.3% Tween 20, followed by three times for 5 min each in PBS plus 0.1% Tween 20. Immunoreactive bands were visualized by use of enhanced chemiluminescence (ECL) Western blotting detection reagents (RPN 2106; Amersham, Arlington Heights, IL).

Electrophysiology

Methods described previously (Karunanithi et al., 1999) were used to record and analyze synaptic currents from individual Ib boutons of motor neuron RP3 innervating muscle 6 of segment 3. Briefly, larvae were dissected in chilled Schneider's solution to prevent contractions and pinned to expose the body wall muscles. The larval brain was removed. Experiments were conducted in HL3 solution (Stewart et al., 1994).

Recordings were made over a wide range of test temperatures (22, 27, 31, 35, and 39°C). The preparation was viewed with a 40× water immersion lens using Nomarski optics and the images were projected onto the computer screen using a low-light intensity video camera (Panasonic WV-BP310) mounted on the upright microscope. In the present experiments, one synaptic bouton was recorded from and analyzed in each larval preparation, thus, 'n' represents the number of boutons independently sampled.

The focal macropatch electrode used to record synaptic currents had its tip opening (~5 μm) manufactured to enclose the chosen bouton. The recorded currents were amplified using an Axoclamp 2A amplifier (Axon Instruments, Foster City, CA) in bridge mode. The same computer used for visualization of the nerve terminals (Apple Power Macintosh 7500/100) was used simultaneously for data acquisition. The MacLab/4S data acquisition system (AD Instruments) was used to record the electrical signals. Evoked responses were elicited at 1 Hz and 300 events were recorded at each test temperature.

The Igor Pro 3 (Wavemetrics) software package was used for data analysis. Appropriate statistical tests were applied to the data using commercial software (Sigmastat; Jandel Scientific, Corte Madera, CA) and significance was assessed at $P < 0.05$. The two-way ANOVA test was used to assess significant differences between nonheat-shocked and heat-shocked groups in *cisII* and *traII* lines and the effects of heat shock treatment and temperature within a fly line. Pairwise comparisons between two groups were made using a *t*-test.

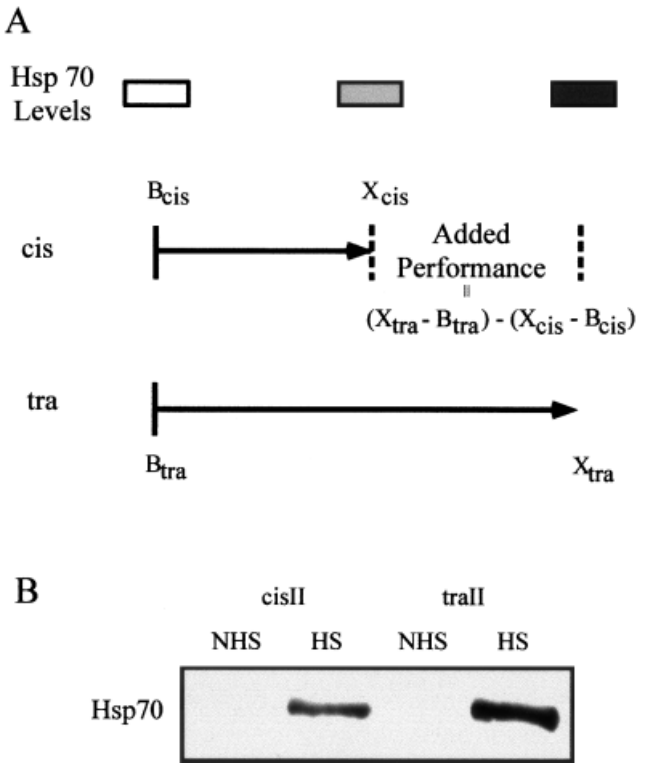

Fig. 1. **A:** Schematic illustration of the quantity defined as "Added Performance" accompanying extra Hsp70 protein in the *traII* line over the *cisII* line following a heat shock (top column designated 'Hsp70 Levels'). B_{cis} and B_{tra} designate the basal values of the synaptic parameter under consideration in the *cisII* and *traII* lines, respectively, in the absence of a heat shock when Hsp70 is not detectable. X_{cis} and X_{tra} represent the values of the synaptic parameters in *cisII* and *traII* larvae, respectively, corresponding to the levels of Hsp70 expressed in each line. **B:** Western blots showing greater amounts of Hsp70 protein produced in *traII* compared to *cisII* larvae following a heat shock (at 36°C for 1 h) and recovery at 25°C for 30 min. NHS, nonheat-shocked (larvae not exposed to prior heat shock); HS, heat-shocked (larvae exposed to prior heat shock).

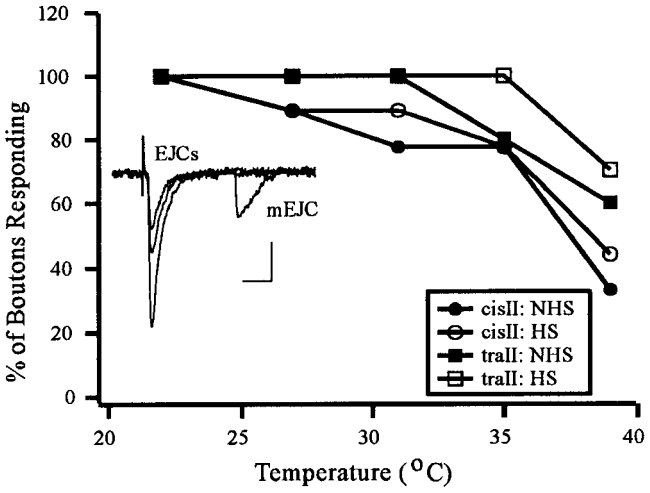

Fig. 2. Selective overexpression of Hsp70 in transgenic animals marginally increased synaptic thermotolerance. Inset: Raw traces of EJCs preceded by the stimulus artifact at 22°C. Also shown is a trace of an mEJC. Scale bars: 0.5 mV (vertical) and 10 ms (horizontal). The graph shows results for nonheat-shocked (NHS; filled symbols) and heat-shocked (HS: open symbols) neuromuscular preparations for both *cisII* (circles) and *traII* (squares) lines. The percentage of boutons responding is shown as a function of temperature in nonheat-shocked (*cisII:* $n = 9$; *traII:* $n = 10$) and heat-shocked preparations (*cisII:* $n = 9$; *traII:* $n = 10$).

Assessing the effects of overexpressing Hsp70

A simple analytical test was used to assess the impact on synaptic parameters of transgenic overexpression of Hsp70 (Fig. 1A). B_{cis} and B_{tra} represent the values of assessed synaptic parameters in *cisII* and *traII* lines, respectively, in the absence of prior heat shock. X_{cis} and X_{tra} represent the corresponding values of the same synaptic parameters following a prior heat shock. The difference, X-B, for either line represents the effect of prior heat shock on synaptic performance. From this, the extent of added performance accompanying overexpression of Hsp70 in the *traII* line, in comparison with the *cisII* line with normally expressed Hsp70 levels, can be represented as:

$$\text{Added performance} = (X_{tra} - B_{tra}) - (X_{cis} - B_{cis}) \quad (1)$$

Alternatively,

$$\text{Added performance} = (X_{tra} - X_{cis}) - (B_{tra} - B_{cis}) \quad (2)$$

Added performance can be deduced at each test temperature for the synaptic parameter being tested.

RESULTS

Expression of Hsp70 at raised temperatures

Elevated temperatures induce greater amounts of Hsp70 protein in *tra* larvae than in *cis* larvae (Feder et al., 1996). We confirmed this result with Western blot analysis of intact larvae, comparing nonheat-shocked specimens with those subjected to prior heat shock (Fig. 1B).

Synaptic thermotolerance

We compared synaptic performance in *cis* and *tra* larvae with and without prior heat shock to assess whether synaptic performance at high test temperatures differs in the two lines. A focal macropatch electrode was used to record both spontaneous and evoked postsynaptic currents resulting from transmitter release at individual, visualized boutons of the larval neuromuscular junction (Fig. 2). We assessed the temperature sensitivity of synaptic transmission by monitoring the percentage of transmitting boutons (those generating evoked or spontaneous events) in nonheat-shocked and heat-shocked preparations as the test temperature was increased (Fig. 2). The overall success rate of synaptic transmission at each test temperature is given by the percentage of transmitting boutons (Karunanithi et al., 1999). In all cases, more than 78% of the boutons responded up to temperatures of 35°C (Fig. 2). Prior heat shock did not significantly improve the

percent of boutons responding for either line, as their responses were fairly robust even in the absence of heat shock. Overexpression of Hsp70 following a heat shock marginally improves the percent of boutons responding in the *traII* line.

Overexpression of Hsp70 does not enhance postsynaptic performance

In the absence of nerve stimulation, spontaneous release of transmitter generates miniature excitatory junctional currents at synaptic boutons (mEJC; Fig. 2, inset). Previous work showed that mEJC amplitude (quantal size) increased and was more variable at higher temperatures in nonheat-shocked controls, whereas following a heat shock, quantal size was stabilized and remained unchanged from room temperature up to 35°C (Karunanithi et al., 1999). For both *cisII* and *traII* lines, mEJC amplitude was plotted as a function of temperature (Fig. 3). To ascertain whether heat shock stabilizes the postsynaptic response, we normalized the mEJC amplitudes at the higher temperatures to those at 22°C for both the *cisII* and *traII* lines (Fig. 3A,B) (Karunanithi et al., 1999).

Comparison between nonheat-shocked *cisII* and *traII* groups (Fig. 3A) revealed no significant differences ($P = 0.877$), and no effects of temperature ($P = 0.0913$). Surprisingly, comparison between heat-shocked *cisII* and *traII* groups (Fig. 3B) also revealed no significant differences ($P = 0.915$), or effects of temperature ($P = 0.405$). The added performance graph (Fig. 3C), constructed from Equation 1, revealed no added postsynaptic improvement linked to overexpression of Hsp70, since $B_{cis} \cong B_{tra}$ and $X_{cis} \cong X_{tra}$.

Presynaptic performance improves with overexpression of Hsp70

Next we assessed whether overexpression of Hsp70 could improve presynaptic performance. Neuromuscular transmission is effected by simultaneous release of synaptic vesicles from several boutons. At a single bouton, nerve stimulation evokes excitatory junctional currents (EJCs), each of which is produced by a variable number of released transmitter quanta (Fig. 2, inset).

The percent success of transmission (Fig. 4A) represents the number of stimuli in a train which evoke the release of at least one quantum of transmitter from a single bouton. Previous work indicated that the percent success following a heat shock correlates with the levels of Hsp70 expressed (Karunanithi et al., 1999). We assessed whether overexpression of Hsp70 in the *traII* line is linked with greater thermotolerance. Comparison between nonheat-shocked *cisII* and *traII* groups (Fig. 4A) showed no significant differences ($P = 0.637$). Effects of temperature on both groups were significant ($P < 0.001$). Heat-shocked *traII* preparations had significantly higher success of transmission

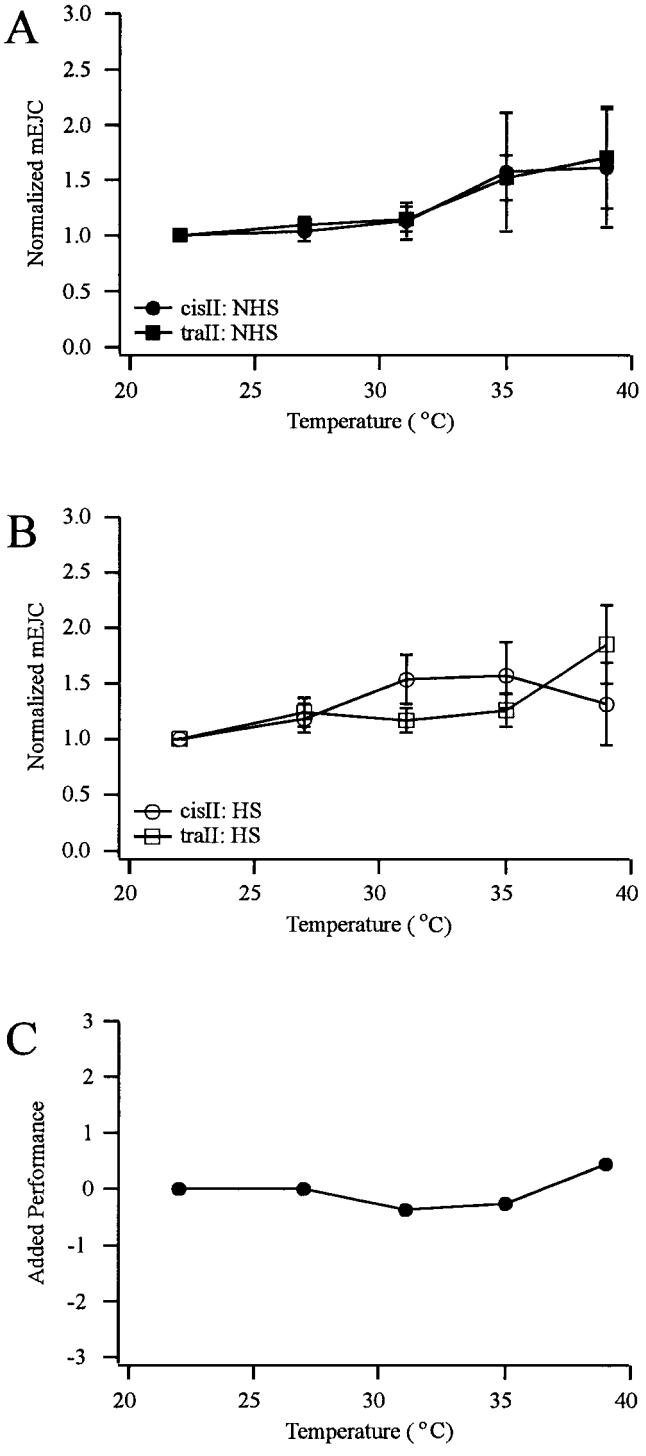

Fig. 3. Overexpression of Hsp70 does not alter postsynaptic stabilization of mEJC amplitudes. The normalized values of mEJC amplitude are shown as a function of temperature for nonheat-shocked (**A:** NHS) and heat-shocked (**B:** HS) preparations (circles: *cisII,* $n = 9$; squares *traII:* $n = 10$). **C:** The added performance graph is constructed from the normalized mEJC graphs in **A** and **B.**

than heat-shocked *cisII* preparations (Fig. 4B; $P = 0.0434$), and both groups showed a significant effect of temperature ($P < 0.001$). By constructing the added

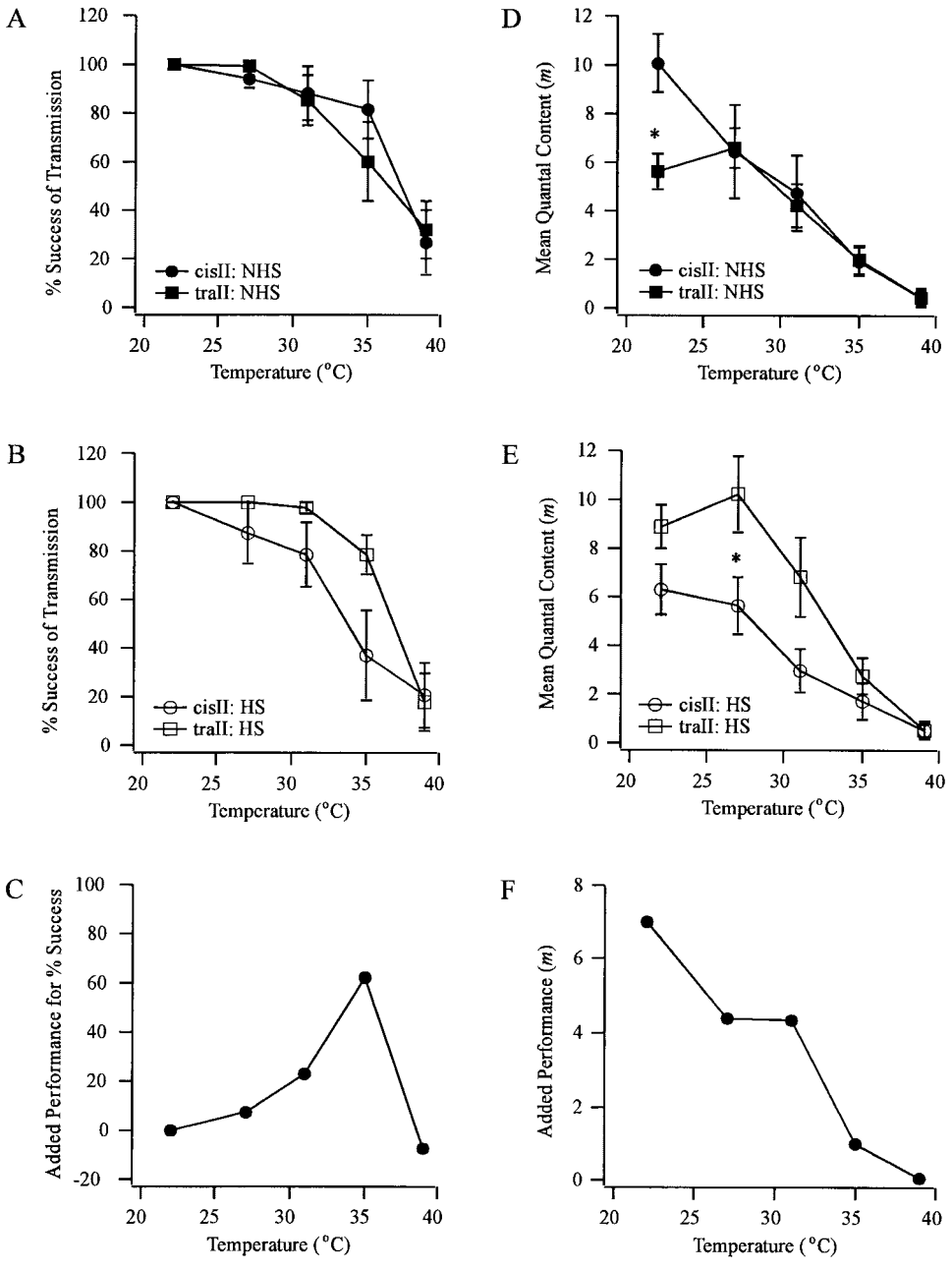

Fig. 4. Overexpression of Hsp70 improves presynaptic performance. For individual boutons, the percent success of transmission (**A:** NHS preparations; **B:** HS preparations) and mean quantal content (**D:** NHS preparations; **E:** HS preparations) are shown as a function of temperature. The extent of added performance for each parameter is also shown as a function of temperature (**C:** percent success; **F:** mean quantal content). Values are shown for both *cisII* (circles, $n = 9$) and *traII* (squares, $n = 10$).

performance curves using Equation 1, we assessed whether overexpression of Hsp70 was beneficial to synaptic performance (percent success) at high temperatures. Since there was no significant difference between the nonheat-shocked *cisII* and *traII* groups, $B_{cis} \cong B_{tra}$ and Equation 1 simplifies to $(X_{tra} - X_{cis})$. Overexpression of Hsp70 in the *traII* line resulted in added performance (Fig. 4C). In contrast, synaptic transmission was less robust in *cisII* larvae expressing normal levels of Hsp70 following a heat shock, up to temperatures of 35°C.

The mean quantal content m (Fig. 4D,E) represents the average number of vesicles released per stimulus in a train. We assessed whether overexpression of Hsp70 induced by prior heat shock resulted in added performance for m. Comparisons between nonheat-shocked *cisII* and *traII* lines (Fig. 4D) revealed no significant differences between the two groups ($P = 0.134$); however, significant effects of temperature ($P < 0.002$) were observed. Surprisingly, pairwise comparisons of m showed that at 22°C the nonheat-shocked *cisII* group displayed significantly larger values ($P = 0.016$) than the *traII* group (Fig. 4D). In heat-shocked preparations (Fig. 4E), significantly larger values of m were found for *traII* in comparison with *cisII* ($P < 0.001$). For both groups, significant effects of temperature ($P < 0.001$) were observed. Pairwise comparisons revealed a significantly larger m at 27°C for the heat-shocked

traII group ($P = 0.0342$). Construction of the added performance graph using Equation 1 (Fig. 4F) revealed that overexpression of Hsp70 provided extra protection for mean quantal content. The large value of added performance at 22°C (Fig. 4D) is due to $B_{cis} > X_{cis}$ in Equation 1.

Overall, overexpression of Hsp70 induced in the *traII* line by a prior heat shock resulted in greater presynaptic thermoprotection (Fig. 4), but had no discernible postsynaptic effect (Fig. 3). Normal levels of Hsp70 expression in the *cisII* line seem sufficient for postsynaptic stabilization. The major effect of Hsp70 overexpression was to maintain a higher quantal release per bouton for temperatures up to 35°C.

DISCUSSION

In *Drosophila,* Hsp70 is the most prominent induced heat shock protein (Parsell and Lindquist, 1993). Its role in protecting cells against thermal injury and apoptosis has been established in studies of cell cultures (Sharp et al., 1999) and mammalian retinal receptors (Barbe et al., 1988; Tytell et al., 1994). The present results indicate a novel role for Hsp70 in promoting and protecting synaptic transmission under adverse conditions. Synaptic performance (quantal release per synaptic bouton) is improved at high test temperatures in transgenic *Drosophila* larvae which overexpress Hsp70. Overexpression of Hsp70 can reduce activities of at least some metabolically important enzymes (Krebs and Holbrook, 2001), but the beneficial effects of Hsp70 for *Drosophila* neuromuscular junctions apparently outweigh such inhibitory effects. By extending synaptic thermotolerance, Hsp70 may in turn permit the intact nervous system to remain functional at high temperatures. In fact, prior heat shock has been shown to protect critical neural circuits in locusts upon subsequent exposure to high temperatures (Dawson-Scully and Robertson, 1998). Enhanced synaptic transmission due in part to Hsp70 induction is likely to contribute to functional improvement.

Presynaptic function is selectively enhanced in transgenic *Drosophila* larvae overexpressing Hsp70, as evidenced by the greater percentage of successes in transmission and higher mean quantal content at high test temperatures. Overexpression did not enhance postsynaptic stabilization, since quantal size was not significantly different between *cisII* and *traII* larvae following heat shock. These results indicate that normal Hsp70 levels may not be sufficient to maximize presynaptic performance at elevated temperatures. Given the short time over which heat shock induced protection appears at *Drosophila* neuromuscular synapses, it is likely that transcription of hsp70 genes in motor neurons is not part of the immediate protective mechanism, since axonal transport of induced protein down relatively long axons to synapses would be necessary. Rapid induction of Hsp70 in muscle and in glial cells proximal to the neuromuscular synapse is clearly evident in heat-shocked *Drosophila* preparations (Brown et al., 1999), indicating the possibility that nonneuronal perisynaptic cells contribute to synaptic protection. Overexpression of Hsp70 in these structures may contribute to greater presynaptic protection. Muscle nuclei in close proximity to the neuromuscular junction rapidly accumulate large amounts of Hsp70. Hsp70 antibodies also detect the protein throughout the cytoplasm of the muscle cells following heat shock (Brown et al., 1999). Postsynaptic expression of Hsp70 could exert transynaptic effects on the presynaptic nerve terminals through one or more retrograde messengers. Several intermediary second-messenger systems are known to act trans-synaptically (Ottersen et al., 1998). Recent evidence suggests that stress-induced Hsp70 associates with synaptic elements in the mammalian brain (Bechtold et al., 2000), but the physiological consequences for synaptic transmission (including pre- or postsynaptic enhancement) have not yet been elucidated.

Mechanisms by which Hsp70 confers synaptic protection, suggested by other investigations, include preservation of second-messenger-mediated cellular pathways, especially those involving cAMP (Zensho et al., 1998); inhibition of protease dependent events; and preservation of synaptic vesicle proteins (Sharp et al., 1999). Overexpression of Hsp70 may reinforce one or more of these protective effects.

Overexpression of Hsp70 did not significantly extend the temperature range for synaptic performance upward; in both *traII* and *cisII* lines, transmission at individual boutons was almost eliminated at 40°C (Fig. 4). Thus, another factor (possibly ATP production) becomes limiting at this temperature and synaptic performance at 40°C of individual boutons cannot be rescued effectively in the short term by overexpression of Hsp70. This illustrates further that thermal dependence of synaptic transmission depends on several metabolic pathways.

In conclusion, the cumulative evidence indicates that Hsp70, a key component of the thermal stress response, does confer neuroprotection to synapses by improving synaptic transmission up to 40°C. Selective overexpression of Hsp70 in transgenic animals exerts its main effect at presynaptic sites. This may help to sustain the survival of organisms under stressful conditions (Feder et al., 1993; Feder and Hofmann, 1999; Barclay and Robertson, 2001). This result has implications for generalized problems of synaptic transmission following heat stress, ischemia, and stroke, and could point the way towards strategies for treatment of such conditions (Yenari et al., 1998).

In response to a range of stressful stimuli, organisms trigger the highly conserved heat shock or stress response. A set of heat shock proteins are induced that are thought to play roles in cellular repair and protec-

tive mechanism. However, very little is known of the functional significance of stress-induced heat shock proteins in the brain or any other organ. The present study demonstrates that selective overexpression of Hsp70 elicits specific effects at the physiological level in the nervous system, namely, enhancement of presynaptic performance.

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