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Stress-Induced Thermoprotection of Neuromuscular Transmission[1]

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SYNOPSIS. Environmental stresses such as high temperature or low levels of oxygen can lead to structural destabilization of cells, disruption of cellular processes, and, in extreme cases, death. Previous experience of sub-lethal stress can lead to protection during a subsequent stress that may otherwise have been lethal. Synapses are particularly vulnerable to extreme environmental conditions and failure of function at this level may be the primary cause of organismal death. Prior heat shock induces enhanced thermotolerance at neuromuscular junctions in the locust extensor tibiae muscle and in abdominal muscles of larval *Drosophila*. Synaptic thermoprotection is associated with an increase in short-term plasticity at these synapses. Prior anoxic coma in locusts induces synaptic thermotolerance suggesting that the same protective pathways are activated. It is well established that diverse forms of stress induce the upregulation of cellular chaperones (heat shock proteins; HSPs) that mediate acquired protection. The mechanisms underlying HSP-mediated synaptic protection are currently unknown but evidence is accumulating that stabilization of the cytoskeleton may play an important role.

INTRODUCTION

The ability of an organism to survive, prosper, and propagate relies on its capacity to sense its environment and to carry out target-oriented tasks specific to its needs. This must be done in a timely, efficient, and appropriate manner in an ever changing environment. Whether the task is to avoid predators, obtain food, or reproduce, the nervous system is responsible for the input of information, the processing of that information, and the motor output required to achieve the behaviour necessary to complete the task at hand successfully. Environmental conditions can greatly affect the ability of the nervous system to carry out its tasks and insect nervous systems operate under widely varying body temperatures, fluctuations that are considered pathological in mammals.

To reduce the stress of extreme environmental conditions animals display a wide array of behavioural responses such as thermal stress avoidance (Robertson *et al.*, 1996). In addition to behavioural responses, physiological protective mechanisms are initiated during stress.

Protection from the harmful effects of environmental stress can be induced, for a period of time, after a brief initial stressful insult such as oxidative stress (Dalle-Donne *et al.*, 2001), a heat shock (Parsell *et al.*, 1993), or an anoxic shock (Wu *et al.*, 2002). When stress-induced protective mechanisms are initiated, they allow individual cells and whole organisms to survive bouts of stress that would otherwise have been lethal. Acquired thermotolerance is likely conferred through mechanisms involving upregulated heat shock proteins (Hsps) (Parsell *et al.*, 1993). The mechanism by which protection is conferred, and the sites of action remain to be fully elucidated.

Thermal stress may induce synaptic dysfunction leading to death suggesting synapses as possible points of weakness (Hochachka and Somero, 2002). Respiratory circuit function recorded in the ventral respiratory group is particularly susceptible to hyperthermic stress. As temperature increases respiratory frequency first increases to dissipate heat and then declines and may lead to apnea and death (Tryba and Ramirez, 2003). Synaptic function recorded in the same region is protected against thermal stress by both prior thermal conditioning and exogenous Hsp 70 application (Kelty *et al.*, 2002). The upper temperature limit of neuromuscular transmission is elevated by heat shock in locust extensor tibiae muscle (Barclay and Robertson, 2000) and in body wall muscles of *Drosophila* larvae (Karunanithi *et al.*, 1999). Like acquired thermotolerance, initiation and maintenance of synaptic thermoprotection coincides with the presence of inducible heat shock proteins and substantial evidence suggests that these heat shock proteins are involved in conferring protection (Parsell *et al.*, 1993; Karunanithi *et al.*, 1999; 2002). The proteins with which inducible Hsps interact give an indication of where and how protection is likely to be mediated.

Various members of the Hsp family interact with elements of the cytoskeleton. These include Hsp 70 (Collier and Schlesinger, 1986) and Hsp 27 (Mounier and Arrigo, 2002) and Hsps have been shown to protect against stressinduced cytoskeletal disruption (Dalle-Donne *et al.*, 2001).

The following review will show how parameters of neuromuscular transmission are altered during transient increases in temperature and how physiological protective mechanisms may act to confer protection against extreme stress. Discussion will focus on the cytoskeleton as a possible target of protective mechanisms and future target of research.

SYNAPTIC THERMOPROTECTION IN *LOCUSTA MIGRATORIA* EXTENSOR TIBIAE MUSCLE

Locusta migratoria can endure harsh conditions, such as ambient temperatures in excess of 45°C, at

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which normal neuronal and synaptic function can be compromised. A prior heat shock can enhance thermotolerance of neuromuscular transmission during a subsequent hyperthermic stress. Heat shock elevates the upper temperature limit of transmission, and stabilizes excitatory junctional potential (EJP) amplitude and duration during an increasing temperature ramp (Barclay and Robertson, 2000). The time taken for synaptic transmission to recover following removal of the stress is reduced. To examine if heat shock-induced synaptic thermoprotection could also be mediated through contractile mechanisms in the muscle, comparisons were made between muscle contractions initiated through nerve stimulation, and those initiated through direct stimulation of the muscle. The upper temperature limit of successfully stimulating a muscle contraction in control animals is higher when neuromuscular transmission is bypassed and the muscle is directly stimulated (Barclay and Robertson, 2000). Therefore, heat shock can increase the failure temperature of nerve stimulated contractions to levels seen when contractions are induced directly. However, heat shock does not lead to a further increase in failure temperature of contractions when muscles are directly stimulated (Barclay and Robertson, 2000). This indicates that synaptic thermoprotection is being conferred by mechanisms at the synapse and not on contractile mechanisms. Further dissection of the sites for protection come from *Drosophila* studies.

SYNAPTIC THERMOPROTECTION IN *DROSOPHILA* LARVA ABDOMINAL WALL MUSCLES

$DiOC_2(5)$ staining of *Drosophila* larva body wall muscles allows the visualization of, and macropatch-electrode placement on, type Ib boutons in order to assess the effects of temperature on synaptic currents (Karunanithi *et al.,* 1999).

Synaptic currents occur from the release of vesicles of transmitter termed quanta. Responses resulting from nerve-stimulated release of numerous vesicles are termed excitatory junctional currents (EJCs). Responses ensuing from the spontaneous release of single quanta are termed miniature excitatory junctional currents (mEJCs). Synaptic current recordings can give indications of both pre- and post-synaptic changes in neuromuscular transmission.

mEJC amplitude is the direct result of the number of receptors activated, the channel open time, and the conductance of each channel. As temperature increases, the amplitude of a quantum increases significantly, doubling and even tripling. A prior heat shock stabilizes mEJC amplitude resulting in no significant change across the tested temperature range. While the rise time and decay constant of mEJCs decrease with increasing temperature, heat shock does not significantly alter either of them (Karunanithi *et al.,* 1999). This suggests that progressive effects on receptor kinetics such as receptor desensitization are not responsible for differences between heat shock and control. Desensitization could, however, still be involved in the end result that is transmission failure. Desensitization of a signal is the reduction of a response resulting from prolonged or repeated activation. Numerous types of densensitization exist, including internalization of receptors, decrease in channel open time, and decrease in gene transcription (Lohse, 1993). How can the temperature-induced increase in Mejc amplitude and the heat shock induced stabilization be explained?

If receptor kinetics are indeed not involved, then heat-shock induced stabilization of mEJC amplitude could result from either a stabilization of the amount of transmitter released and/or a stabilization of the receptor population in the postsynaptic density. No change in vesicle size was observed as a result of heat shock (Karunanithi *et al.,* 1999). Vesicle size is believed to be correlated with the amount of transmitter contained and the amplitude of quantal events in some synapses (Wilson and Frerking, 1998), however the effect of heat shock on vesicle transmitter concentration has not been investigated to this date. Receptor populations can be altered in the short term resulting in significant effects on response parameters. Various glutamate receptors have been shown to redistribute away from the synapse within as little as 5 minutes under certain stimuli (Carroll *et al.,* 1999; Lissin *et al.,* 1999; Lu *et al.,* 2001). Temperature has been shown to alter membraneembedded protein endocytosis (Shah *et al.,* 2002), and in HeLa cells temperature sensitive mechanisms involving microtubules mediate pathways involved in mannose 6-phosphate/insulin like growth factor II receptor endocytosis (Waguri *et al.,* 2003). Although temperature has not been tested as a stimulus for redistribution of glutamate receptors, it could play a part in the observed progressive decrease in amplitude of nerve stimulated EJPs and EJCs during an increasing temperature ramp. If this were indeed the case, how could the increase in mEJC amplitudes be explained? Since it is likely that one quantum of transmitter does not activate all of the available receptors in the post-synaptic density (Mainen *et al.,* 1999; Conti and Lisman, 2003), the amplitude of mEJCs might rely less on receptor density and more on parameters such as channel conductance, which typically increases concomitantly with temperature (Stettmeier *et al.,* 1983).

Absence of a detectable response to a nerve stimulation is termed a failure. As temperature increases, failures begin to occur. However, in heat shocked preparations failures do not occur until the final test temperature. Even at this high temperature (36°C) failures are far fewer in heat shocked animals. While the number of boutons successfully transmitting neural activity above room temperature is increased by heat shock, those boutons which do successfully transmit activity, whether they are control or heat shock, do not show altered frequency of spontaneous release (Karunanithi *et al.,* 1999). Thus, the increased release of transmitter in heat shocked preparations is the result of increased evoked release and not the result of increased spontaneous release adding to the evoked signal.

EJCs in *Drosophila,* like EJPs recorded in locust

muscle, decline in amplitude as temperature increases. Heat shock treatment attenuates this progressive decline thus protecting amplitude. At room temperature the amount of transmitter released is not different between heat shock and control preparations. As temperature increases though, heat shocked animals release more evoked (nerve-stimulated) transmitter (Karunanithi *et al.*, 1999).

Overexpressing Hsp 70 levels leads to enhanced pre-synaptic thermotolerance. Level of protection can be scored by the number of boutons able to transmit signals at high temperatures. Engineered flies overexpressing Hsp 70 (traII) after heat shock show an increased number of boutons successfully transmitting evoked signals compared to their control line (cisII) (Karunanithi *et al.*, 2002). After heat shock, neuromuscular transmission in traII flies is not affected as greatly by increasing temperature, and release of transmitter is enhanced above room temperature (Karunanithi *et al.*, 2002). TraII mutants have a greater proportion of boutons which can generate either an EJC or a mEJC, compared to cisII. TraII and cisII show no difference in quantal size (amplitude of mEJCs) revealing that overexpression of Hsp 70 has no post-synaptic effect (Karunanithi *et al.*, 2002). Since heat shock confers post-synaptic protection in the wild-type *Drosophila* larvae (Karunanithi *et al.*, 1999) other Hsps are likely involved post-synaptically.

It has been suggested that the decline in EJC amplitude as temperature increases is the result of the decline in the amount of evoked transmitter released (pre-synaptic) overcoming the concomitant increase to the post-synaptic response seen in mEJCs amplitude (post-synaptic) (Karunanithi *et al.*, 1999). Further investigations are needed to clarify if heat shock exerts any effects on receptor trafficking and/or vesicle transmitter concentration.

HEAT SHOCK AND SYNAPTIC PLASTICITY

Heat shock affects neuromuscular transmission from locust slow extensor tibiae (SETi) motoneurons. Effects of heat shock include increased short term facilitation of EJPs and decreased amplitudes at all temperatures tested compared to controls (Barclay and Robertson, 2001). However, in both heat-shocked and control preparations, EJPs showed an increase in amplitude as temperature increased. Contrary to this, amplitude of synaptic responses decrease as temperature increases in locust FETi preparations (Barclay and Robertson, 2000) and in *Drosophila* type 1b boutons preparations (Karunanithi *et al.*, 1999). Type 1b boutons in *Drosophila* are the equivalent of the slow neuromuscular junctions in locust, yet effects at these synapses are more representative of effects seen at locust fast junctions. This suggests that generalizations on slow and fast synapses cannot be made across species.

Effects of heat shock on plasticity could be the result of altered passive membrane properties. Both the time constant of decay and the input resistance are increased after a heat shock, however the physiological

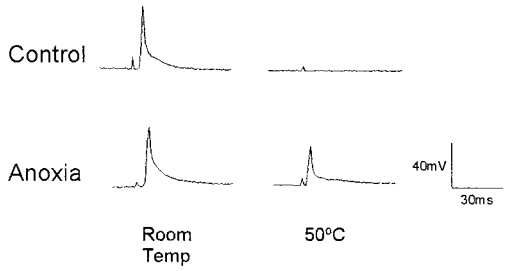

FIG. 1. Excitatory junctional potentials recorded intracellularly in extensor tibiae muscle. EJP traces from control and anoxia-treated preparations at room temperature and at 50°C. Stimulus artifacts are visible a few milliseconds before the much larger EJP.

significance of each is argued against (Barclay and Robertson, 2003). An increased time constant of decay implies that heat shock increases EJP duration, however, this is not evident. Using similar reasoning, increased input resistance would necessitate a compensatory reduction in current, because unlike locust EJP amplitude, *Drosophila* EJP amplitude is not altered by a heat shock. Since the synaptic currents (EJCs) in *Drosophila* increase in amplitude, it is argued that input resistance is not relevant (Barclay and Robertson, 2003). However, amplitude of mEJCs do decrease after heat shock and could well be a compensatory reaction to balance a possible significant effect of the increased input resistance. Therefore, the effects of passive membrane properties on synaptic plasticity remain unclear.

Elevating saline calcium concentration enhances the thermal operating limit of *Drosophila* synapses in control animals to levels attained after heat shock and also increases the amplitude of EJPs across the temperature range as seen in heat shocked locust EJPs (Barclay and Robertson, 2003). Since synaptic plasticity has been linked to pre-synaptic calcium handling (Zucker and Regehr, 2002; Regehr and Tank, 1994; Meinrenken *et al.*, 2003), the effects of heat shock are believed to be, in part, the result of residual calcium in the pre-synaptic terminals (Barclay and Robertson, 2003). Calcium imaging techniques could clarify whether heat shock has an effect on calcium handling.

CROSS-TOLERANCE

Heat shock has also been shown to induce protection against other stresses such as anoxia, excitotoxicity and apoptosis. We tested for cross-tolerant synaptic thermoprotective effects by performing a hyperthermic stress test on locust neuromuscular junctions after an anoxic shock. Locusts were exposed to 100% nitrogen for 2 hours. Within the first two minutes locusts go into a hypoxic coma. Returning the locusts to normoxic conditions resulted in emergence from coma within approximately thirty minutes. Locusts were allowed to recover for 2–6 hours. EJPs were then recorded in the fast extensor tibiae preparation (Hoyle and Burrows, 1973), during an increasing temperature stress (Fig. 1). All experiments were performed on the

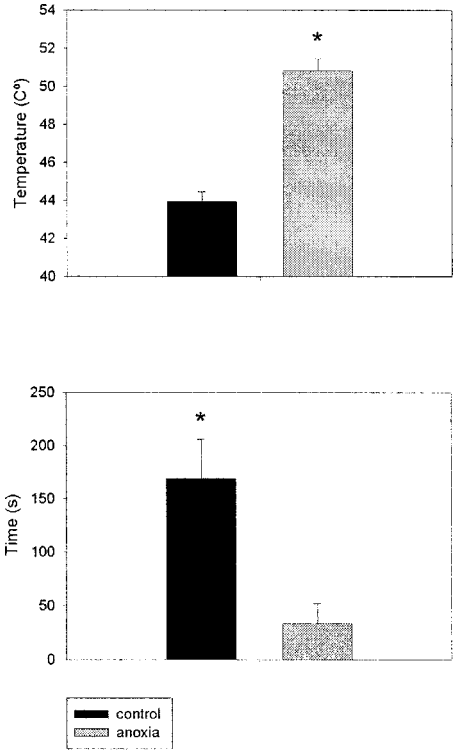

FIG. 2. Anoxic shock induced thermoprotection of synaptic transmission. A) Exposure of locusts to a two hour anoxic shock resulted in an elevation of the temperature at which synaptic transmission failed. B) Post failure recovery time of synaptic transmission was decreased by anoxia treatment. Values shown are means ± standard error mean. Significance determined using a t-test and indicated using an asterisk (*).

metathoracic extensor tibiae muscle of adult *Locusta migratoria*, 2–5 weeks post imaginal molt, using intracellular recording techniques and Axoscope data analysis software. FETi motorneurons were stimulated by severing nerve 5 of the metathoracic ganglion and stimulating it with a suction electrode with a square pulse (duration = 0.5 ms; frequency = 1 Hz). Locust saline composed of (in mM/L) 147 NaCl, 10 KCl, 4 CaCl2, 3 NaOH, 10 HEPES (pH 7.2) was perfused over the muscle and heated at 5°C/minute. Heat was turned off when synaptic failure occurred, however saline perfusion continued until recovery of the response occurred or 10 minutes had passed. Saline temperature was monitored using a copper constantan thermocouple (0.2 mm diameter, Bat-12, Sensortek, Clifton, NJ, USA).

Anoxic shock increased the upper temperature limit of neuromuscular transmission by 6.7°C in the fast extensor tibiae muscle of *Locusta migratoria* from 44°C to 51°C (*t*-test, $t = 7.67$, $P = 0.001$, df = 56) (Fig. 2A). Synaptic recovery time, or the time required for the neuromuscular junction to resume a noticeable response at least twice the amplitude of noise, decreased significantly from 169 ± 37 s to 34 ± 18 s as a resuult of anoxic shock (Fig. 2B). The temperature induced decrease of EJP amplitude amplitude was reduced by anoxic pretreatment (Fig. 3A). A two-way ANOVA revealed a significant effect of increasing temperature on EJP amplitude ($F = 124.801$, $P < 0.001$, df = 7) and a significant effect of anoxia treatment ($F = 55.45$, $P < 0.001$, df = 1). Furthermore, significant protective effects were conferred by anoxic treatment against the

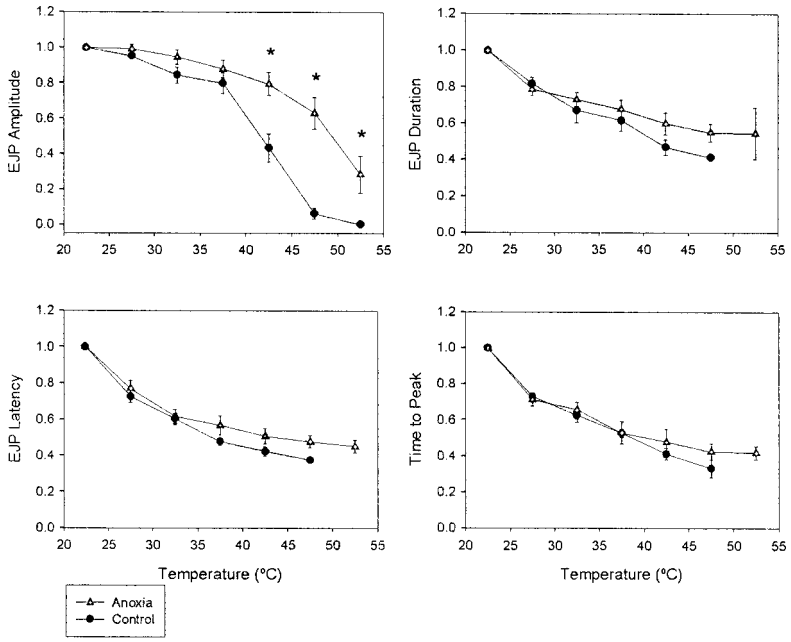

FIG. 3. Anoxia induced effects on excitatory junctional potential parameters. A) Sensitivity of temperature dependent decreases in amplitude was reduced by anoxic treatment. Three individual temperature bins showed significantly different EJP amplitudes between treatments, 40–45°C (*t*-test, $t = 2.54$, df = 21), 45–50°C (Mann-Whitney, $t = 192$, $P = .001$), and 50–55°C (Mann-Whitney, $t = 160$, $P = .041$). B) Normalized latency. C) Normalized duration, taken at half the maximal amplitude. D) Normalized time to peak. Values shown are means ± standard error mean. Significance determined using a t-test and indicated using an asterisk (*).

detrimental effects of increasing temperature at high temperatures ($F = 8.39$, $P = 0.001$, $df = 7$). No effect was seen on other EJP parameters *e.g.*, latency, duration at half amplitude, and time to peak (Fig. 3B, C, D). These experiments reveal that anoxia induces the same protective effects as those arising from prior heat shock. These include an increase in the operating range of neuromuscular transmission, a stabilization of response amplitude as temperature increases, and an increased rate of recovery following the termination of stress. However, one difference between the effects of heat shock and anoxia was seen. Duration at half maximal amplitude was not altered significantly by a prior anoxic shock such as has been seen resulting from heat shock (Barclay and Robertson, 2000). However, a similar trend was evident and it is difficult to determine equivalence of the magnitude of stress applied to the system during heat shock and anoxia treatments. The data support the idea that various stressors can activate equivalent protective pathways. The common trigger which activates the protective pathways is still unclear, however it has been suggested that the presence of denatured proteins arising during an initial stress are responsible (Parsell *et al.*, 1993).

STRESS PROTECTION AND A ROLE FOR THE CYTOSKELETON

Microfilaments, intermediate filaments, and microtubules form the basic structure of cells termed the cytoskeleton. The cytoskeleton can contribute to activity-dependent processes underlying synaptic plasticity through regulation of cellular morphology and signaling proteins. Functions of the cytoskeleton include sculpting cell morphology, exo- and endocytosis, cell division, cell polarity, intracellular migration, intercellular adhesion, signal transduction, and regulation of ion channel populations and activity (Molitoris, 1997). The cytoskeleton is one of the most sensitive and earliest targets of stress and hyperthermic, anoxic, and oxidative stressors can each disrupt the integrity of the cytoskeleton (Dalle-Donne *et al.*, 2001). Stress causes microtubules to disassemble, intermediate filaments to ''cave in'' towards the nucleus, and actin microfilament integrity to disrupt resulting in dissociation from the cell membrane (Loktionova *et al.*, 1999; Molitoris *et al.*, 1997; Dalle-Donne *et al.*, 2001). Interactions between Hsps and the cytoskeleton suggest that elements of the cytoskeleton are likely targets of protective mechanisms. Investigating these interactions will likely provide valuable clues as to how acquired thermotolerance is conferred for an organism. Furthermore, cytoskeletal influences on signalling proteins may shed light on mechanisms involved in synaptic thermoprotection.

Large Hsps such as Hsp 90 and Hsp 70 bind to microtubules, while the smaller Hsps such as Hsp 27 and αβ-crystallin interact with microfilaments (Liang and MacRae, 1997). α-Crystallins stabilize microfilaments, preventing depolymerization as well as aggregation (Stewart *et al.*, 1994). Chemical depolymerization of actin by cytochalasin D can be almost completely blocked by αcrystallin in lens epithelial cells. α-crystallin can also prevent heat-induced aggregation of actin filaments by stabilizing actin polymers (Wang and Spector, 1996). Overexpression of Hsp 27 after a heat shock or oxidative stress results in increased microfilament stability, accelerated recovery, and increased survival rate of cells (Lavoie *et al.*, 1993*a*, *b*; Huot *et al.*, 1995). When cells lacking the ability to express Hsps are exposed to temperatures of 50°C, microtubule disassembly and tubulin inactivation occur (Coss *et al.*, 1982). Heat shock proteins protect the integrity of the cytoskeleton under stress likely mediating, at least partially, stress-induced protection of cells, organs and organisms from subsequent stressors.

Actin microfilaments are thought to be involved in rapid activity-dependent changes in dendrite morphology while microtubules are likely more involved in long term plasticity (Kaech *et al.*, 2001). Rearrangement of the cytoskeleton plays a key role in the processes of neuronal plasticity and destabilizing microtubules and microfilaments disrupts the performance, retention, and repeated acquisition of plastic reactions in *Lymnaea stagnalis* (Zapara *et al.*, 2000). Temperature-induced morphological alterations to the synapse could significantly modulate signal transmission, but how structural stabilization of the synapse could confer protection is unclear. Temperature-induced structural modifications could be responsible for alterations to passive membrane properties such as input resistance. Heat shock has been shown to increase input resistance in *Drosophila* muscle (Barclay and Robertson, 2003), however it has not been tested in locust muscle. An increase in input resistance would result in an increase in EJP amplitude, and would be consistent with heat shocks effect on EJP amplitude in locust muscle.

The cytoskeleton can have significant effects on active properties of the membrane as well. Localization and mobility of glutamate receptors can be manipulated through cytoskeletal disruption (Allison *et al.*, 1998; 2000). Redistribution can be rapid, as seen in prolonged exposure of hippocampal AMPA receptors to glutamate which results in a rapid redistribution of receptors away from the synapse peaking minutes (5–12) after stimulation (Carroll *et al.*, 1999). Temperature has not been tested as a stimulus for glutamate receptor redistribution.

Activation of NMDA receptors, accompanied with a rise in local intracellular calcium, is sufficient to trigger a slow and sustained recruitment of actin into dendritic spines. In contrast, opening of voltage-gated calcium channels rapidly and reversibly enhanced cortical actin at the somatic periphery but not in the spines (Furuyashiki *et al.*, 2002). Conversely, mechanical perturbation of cells can lead to cytoskeletal effects on ion channels leading to, for example changes in intracellular calcium concentrations (Janmey, 1998). Interestingly, interactions between glutamate receptors, the cytoskeleton, and calcium can alter channel kinetics. This is seen in calcium-dependent inactivation of

NMDA receptors where increased intracellular calcium, either directly or by way of calmodulin activation, reduces channel open probability by disrupting interactions with α-actinin, the protein link to actin filaments (Krupp *et al.*, 1999). This reveals the intimate interplay between structural stability, channel activity, and plastic responses of cells.

To test the idea that the cytoskeleton is involved in synaptic thermoprotection we pharmacologically disrupted the actin cytoskeleton to examine if we could interfere with the effects of heat shock. Treatment of muscle (heat shock n = 10; control n = 9) with cytochalasin B (10^{-5} *M*), for 1 hour prior to the experiment, significantly reduces the failure temperature of neuromuscular transmission in heat shocked locusts by 8.53 °C (*t*-test, $t = 2.9$, df = 17, $P = 0.011$) to control levels. Cytochalasin B did not significantly alter the failure temperature of synaptic transmission in control locusts. Taken together these data is consistent with the idea that the mediators of synaptic thermoprotection are exhausted during the cytochalasin B exposure. Perhaps upregulated Hsps are being consumed by the denatured proteins resulting from cytochalasin B exposure leaving insufficient capacity for further protection. Another explanation could be that Hsp function is directly disrupted by cytochalasin B.

CONCLUSION

Hyperthermic, anoxic, and oxidative stressors can each disrupt the integrity of the cytoskeleton, induce the upregulation of Hsps, and induce protection against subsequent stressors. Understanding the effects of hyperthemic stress on neuronal function and protective mechanisms involved in tolerance may be relevant to several clinical conditions including heat stroke and fever, as well as in cases of sudden infant death syndrome where body temperature is found to be elevated upon death.

We have shown that a prior anoxic shock induces synaptic thermoprotection in locust muscle. Stress can cause an increase in denatured proteins and somehow trigger the upregulation of Hsps. These Hsps interact with the cytoskeleton to stabilize structural morphology and may also be responsible for stabilizing synaptic transmission and ultimately conferring enhanced stress tolerance. The mechanisms underlying Hsp-mediated synaptic protection, both post-synaptic and presyntaptic, are currently being unveiled and evidence is accumulating supporting a role for cytoskeletal stabilization in thermotolerance and synaptic thermoprotection alike. We have presented evidence here that elements of the cytoskeleton may be involved in conferring synaptic thermoprotection. The cytoskeleton and the proteins it interacts with such as heat shock proteins are tantalizing targets of future investigations of protection.

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