Thermal stress and neural function: adaptive mechanisms in insect model systems

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Abstract

Neural circuit function is vulnerable to hyperthermic failure but can be protected by stress pretreatments, such as exposure to a brief, sub-lethal high temperature (heat shock, HS), by increasing the time to failure and decreasing the time to recover.

Insects provide excellent model systems to investigate potential mechanisms underlying thermotolerant operation.

Induced thermotolerance is mediated by increased expression of heat shock proteins, HSPs, notably HSP70. Enhanced expression of HSP70 by increasing the gene dosage does not improve HS-induced thermotolerance of larval locomotion or locomotor central pattern generation in *Drosophila*.

Prior stress down-regulates neuronal K^+ currents and this is associated with adaptive increases in the duration of action potentials.

Hyperthermic failure and recovery of the ventilatory central pattern generator in locusts is tightly correlated with a catastrophic increase in extracellular K^+ concentration and its subsequent restoration.

These, and other data, suggest that neural circuit function can be protected by a stress-induced upregulation of HSPs that stabilize the cytoskeleton and preserve the operation of important membrane proteins such as ion channels, receptors and the Na^+/K^+-ATPase.

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1. Introduction

At extreme temperatures, cells and tissues die. Long before this point, however, organisms experiencing hyperthermia are endangered by impaired neural performance that prevents properly coordinated behaviour and hampers the generation of vital motor patterns (Robertson, 2004). Good examples of neural circuits whose impairment would be life-threatening include those for predator detection and avoidance, for effective locomotion to less extreme microenvironments and for the control of ventilatory movements. Furthermore, in humans heat stroke is characterized by central nervous dysfunction that results in delirium, convulsions and coma (Bouchama and Knochel, 2002). Clearly, mechanisms that cope with hyperthermic disturbances to neural circuit operation would be highly adaptive for any organism but are likely to be particularly well-developed in poikilotherms which are more at the mercy of environmental temperature fluctuations. Insects thus provide excellent model systems for the investigation of such neural plasticity because they are poikilotherms

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with experimentally accessible vital neural circuits for which much background information exists in the literature. We use insect model systems exposed to heat shock (HS) pretreatments to determine the mechanisms underlying thermoprotection in neural processes such as action potential generation, synaptic transmission and central pattern generation (e.g. Robertson et al., 1996). In particular we have focused on detection of looming visual stimuli, escape jumping and ventilation in locusts, and larval locomotion in fruit flies.

2. Temperature sensitivity of neural circuits

Neural phenomena under temperature stress demonstrate two main types of sensitivity. One is the immediate sensitivity derived from Q_{10} relationships as is evident in the temperature effects on conduction velocity, action potential duration, synaptic parameters, motor pattern frequency, etc. (Montgomery and Macdonald, 1990; Janssen, 1992; Robertson, 1993; Tryba and Ramirez, 2003). The rates of activation and inactivation of ion channels, for example, are highly temperature-dependent being the result, like enzyme function, of conformational changes in protein structure that are accelerated by increases in temperature. This form of sensitivity does set upper and lower temperature limits for circuit operation when a neuronal parameter is rendered ineffective for proper function. Thus failure of action potential generation at high temperatures is predicted by Hodgkin–Huxley equations (Chapman, 1967) and modulation of ion channel properties, by using different protein isoforms or combinations of subunits, or by phosphorylation, could extend the operating range in either direction. The other form of sensitivity reflects an accumulation of change and thus has a time-dependence. For example, the time taken for a central pattern generator (CPG) to fail when held at a constant extreme high temperature reflects an accumulation of damage or disturbance that eventually crosses a threshold for proper function. For this type of thermosensitivity it is the combination of time and intensity of heat stress that determines the thermal dose the tissue can withstand (Gerner, 1987; Hochachka and Somero, 2002). Prior environmental stress affects both of these types of sensitivity for the phenomena we have been investigating, and one of the most consistent results is that a prior HS or anoxia markedly reduces the time it takes the system to recover from heat-induced failure (from 9 to 1–3 min for ventilatory pattern generation, Newman et al., 2003; 400 to 1 s for FETi neuromuscular transmission, Barclay and Robertson, 2000).

Recently I reviewed the protective effects of prior environmental stress on aspects of neural signaling and circuit operation (Robertson, 2004). Here I will briefly consider candidate mechanisms for mediating this protection: upregulation of protein chaperones and modulation of potassium conductance.

3. Protective mechanisms

3.1. Heat shock proteins (HSPs)

Sub-lethal hyperthermia induces HS responses involving increased expression of a suite of HSPs which results in acquired thermotolerance of organisms (Parsell et al., 1993; Feder and Hofmann, 1999). It is well established that HSPs are involved in physiological stress responses generally (Kregel, 2002) and have roles in mitigating cellular damage in the nervous system (Sharp et al., 1999; Ohtsuka and Suzuki, 2000). Thus there has been mounting interest in the development of therapeutic approaches targeting HSPs (particularly HSP70) for neuronal cytoprotection (Morimoto and Santoro, 1998; Yenari, 2002; Bonini, 2002). Synaptic operation in insects can be thermoprotected by a previous HS (Dawson-Scully and Robertson, 1998; Barclay and Robertson, 2000, 2001; Karunanithi et al., 1999; Klose and Robertson, 2004) and there is evidence that enhanced expression of HSP70 can improve synaptic thermoprotection (Karunanithi et al., 2002) suggesting a mechanistic role for this chaperone in protection. We have also shown in a mouse brainstem slice that HSP70 is sufficient, but not necessary, to mitigate the effects of heat stress on miniature post-synaptic currents in neurons of the pre-Böetzinger complex, which contains the neural circuit for the respiratory motor rhythm (Kelty et al., 2002). Nevertheless cytosolic chaperones such as HSP70 are multifunctional (Young et al., 2003) and it has been equally well established that under certain conditions maintained elevation of HSP70 concentration can be deleterious (Krebs and Feder, 1997, 1998). In an attempt to establish a role for HSP70 in the protection of circuit function, rather than simply neuronal survival, we examined the thermosensitivity of larval locomotion in wild-type *Drosophila* and compared them with flies that had been genetically engineered to have enhanced expression of HSP70 (Welte et al., 1995; Feder et al., 1996).

We assessed locomotor ability in larval *Drosophila* at room temperature (data not shown) and at 42 °C (Fig. 1) by measuring velocity and distance travelled during three consecutive 5 min periods (Klose et al., 2004b). During hyperthermia the path length of control (Canton S) larvae reduced to zero and the ability to locomote was markedly improved by a HS pretreatment (Fig. 1A). We anticipated that, after HS, genetically engineered flies with enhanced expression of HSP70 (12 extra copies of the HSP70 gene; *traII*) would outperform the insertional control flies (*cisII*) at high temperatures.
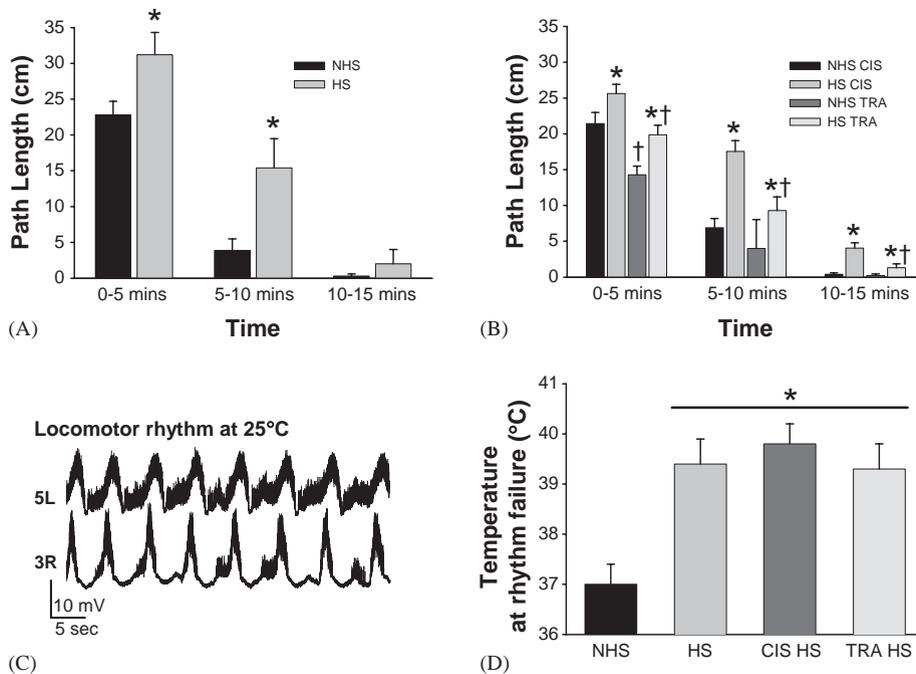
Fig. 1. HS-mediated protection of larval locomotion and locomotor central pattern generation in *Drosophila* is not enhanced by overexpression of HSP70. A. Locomotor path lengths of non-heat shocked (NHS) wandering 3rd instar larvae (Canton S strain) reduce to zero in successive 5 min intervals at 42 °C. After a prior HS path lengths are significantly (asterisks) longer during the first and second 5 min intervals and the larvae are still capable of some locomotion in the third 5 min interval. B. Comparison of locomotor ability at 42 °C in NHS and HS, CIS (insertional control) and TRA (12 extra copies of the HSP70 gene) lines. In each time interval and for both lines there is a significant increase in path length as a consequence of a prior HS (asterisks indicate significant differences between HS and NHS in the different fly lines). There is a significant reduction in path length with overexpression of HSP70 (TRA) compared with the insertional control (CIS) at all time intervals in HS larvae and during the first 5 min interval for NHS larvae (daggers indicate significant differences between CIS and TRA within different pre-treatment conditions). C. Intracellular recordings of patterns of excitatory junction potentials in abdominal muscle 6 on the left side of segment 5 (5L) and on the right side of segment 3 (3R) show the centrally generated locomotor rhythm at 25 °C. D. In NHS Canton S larvae the central locomotor rhythm fails around 37 °C. After a prior HS in Canton S larvae (HS) and in CIS and TRA larvae the temperature at failure is increased to 39–40 °C, however there is no significant difference between CIS and TRA HS larvae (the line above the bars and the asterisk indicate that HS, CIS HS and TRA HS are each significantly different from NHS but are not different from each other). (A and B modified from Chu et al., 2003; C modified from Barclay et al. 2002; D from Klose et al., 2004b).

When assayed at room temperature locomotor ability was identical for all flies (Canton S, *cisII*, *traII*) before and after HS (data not shown). At high temperature, however, the extra-copy flies were impaired relative to the insertional controls both before and after HS (Fig. 1B). We measured the thermosensitivity of the neural circuits for locomotion by recording fictive rhythms produced by the locomotor CPG (Cattaert and Birman, 2001; Barclay et al., 2002; Fig. 1C) and found that the temperature at which rhythm generation fails was increased significantly by a HS pretreatment but this was not enhanced by overexpression of HSP70 (Fig. 1D). These results do not rule out a role for HSP70 in mediating neural thermoprotection, rather they show that the abundance of HSP70 is not limiting for effective protection. Indeed there were indications that the over-expressing line was impaired in its ability to locomote at high temperatures relative to controls. Other studies using these lines have shown that only under particular defined circumstances, if at all, do the *tra* flies outperform the *cis* flies (Le Bourg et al., 2002; Roberts et al., 2003). These are likely periods of extreme hyperthermic stress whereas at other, less stressful, times the extra HSP70 proves a liability (Krebs and Feder, 1997). Moreover desert-dwelling flies, *Drosophila arizonae*, have evolved mechanisms for thermoprotection that are apparently independent of HSP70 (Newman et al., 2004). Using the UAS–GAL4 system for tissue-specific expression without HS of HSP70 and double-stranded HSP70, future experiments will determine whether and how presynaptic and/or postsynaptic HSP70 mediates synaptic and locomotor thermotolerance.

3.2. Modulation of potassium conductance

Neural signalling is dependent on tight temporal and spatial control of ion flow across membranes, and on maintaining the ionic gradients that provide the energy for these currents. As indicated above, activation and inactivation of ion channels is highly temperature-dependent and the failure of action potential generation is predicted by the Q_{10} relationships in the Hodgkin–Huxley equations. Species that are adapted for different temperature regimes have action potentials that differ in their temperature sensitivity (Hodgkin and Katz, 1949) and this results from adaptive changes to the relative strengths of Na^+ and K^+ conductances (Rosenthall and Bezanilla, 2002). Desert-adapted *Drosophila arizonae* have longer duration excitatory junction potentials and this is likely a similar adaptation to enable synaptic function at higher temperatures (Newman et al., 2004). We found that action potentials in flight motoneurons of locusts have longer duration action potentials after HS and after anoxic stress (Wu et al., 2001, 2002) consistent with stress-mediated reductions of whole cell K^+ conductance recorded from neuronal somata in ganglion slices (Ramirez et al., 1999; Wu et al., 2002; Fig. 2). There are at least two important consequences of a reduction in neuronal K^+ conductance: first the increase in action potential duration noted above, and second a reduction in the potential build-up of extracellular K^+ concentration (i.e. loss of the K^+ gradient across the neuronal membrane). To examine the effect on action potentials we use the locust descending contralateral movement detector (DCMD) which is an interneuron that faithfully relays, from the brain to the motor centres in the thorax, vital information about the looming approach of predators in the form of high-frequency bursts of action potentials. HS modulates the excitability of the DCMD axon enabling it to support high-frequency firing at high temperatures without significantly increasing action potential duration (Anstey et al., 2003; Money et al., 2003). These data demonstrate adaptive effects of HS on axonal ion channels but it remains to be determined what mechanisms exist to
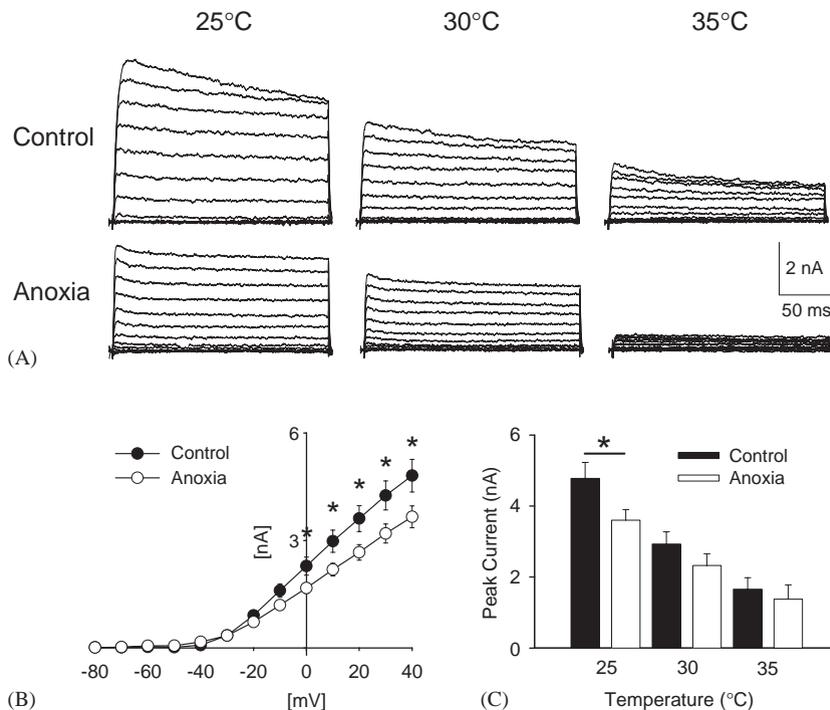

Fig. 2. Prior anoxia reduces whole cell potassium conductances recorded from neuronal somata in a slice preparation of the locust metathoracic ganglion. A. Comparison of whole cell currents in response to step changes in potential to −80 to 40 mV in increments of 10 mV from a holding potential of –60 mV, recorded in preparations taken from control locusts and preparations taken from locusts that had been held in an anoxic coma for 2 h and allowed to recover for 1 h. Outward currents reduce with increasing temperature (25 °C, 30 °C, 35 °C) and prior anoxia reduces the amplitude of the currents at each temperature. B. I/V plots of outward potassium currents in control and anoxia locusts at 25 °C (asterisks indicate significant differences between control and anoxia at indicated voltage steps). Note reduction of the slope of the relationship (conductance) after anoxia. C. Peak currents in response to a step change to 40 mV from a holding potential of −60 mV reduce with increasing temperature and are smaller in anoxia pre-treated preparations (asterisk indicates significant difference between control and anoxia at 25 °C). (A, B and C modified from Wu et al., 2002).

mediate this modulation. To examine the role of extracellular potassium we use the locust ventilatory CPG.

Ventilation is a crucial motor activity to locusts and it is controlled by a robust central pattern generator in the metathoracic ganglion capable of operating even when experimentally isolated (Bustami et al., 2002). It is particularly important during heat stress because the increased ventilatory rate dissipates body heat (Prange, 1990). A relaxed locust shows discontinuous ventilation (Bustami and Hustert, 2000) but under stress ventilation is continuous which makes it easy to determine when the system has failed and when it has recovered. Thus it is an ideal system for investigating vulnerability and protection of neuronal circuits. We measure the thermosensitivity of rhythm frequency, the time it takes to reach failure at a maintained high temperature (around 45 °C), the time it takes the system to recover after normal temperature is restored and the proportion of preparations that fail and recover. Prior stress (HS, cold shock and anoxia) protects the ventilatory CPG against hyperthermic failure and this can be mimicked by an application of serotonin (5-hydroxytryptamine, 5HT) (Fig. 3A). Prior stress also reduces whole cell K^+ currents of neuronal somata (Ramirez et al., 1999; Wu et al., 2002) and this too is mimicked by an application of 5HT (Fig. 3B). Together these data suggest that modulation of K^+ conductance might be the causal link by which 5HT application protects central pattern generation. 5HT does increase action potential duration and synaptic potential amplitude in locusts (Parker, 1995) which would allow more scope for Q_{10}-like temperature-induced reductions of these signals. However this would not easily explain the time-dependent nature of failure and recovery of the ventilatory CPG. An alternative, though not necessarily mutually exclusive, explanation is that failure is associated with the activity- and temperature-induced build-up of extracellular K^+ and consequent loss of the K^+ gradient.

To test this idea we measured extracellular K^+ concentration around the ventilatory CPG in the metathoracic ganglion using a K^+-sensitive microelectrode inserted through the sheath and referenced to an adjacent voltage electrode. Simultaneously we recorded the ventilatory motor pattern using an electromyographic electrode placed on an abdominal expiratory muscle and increased the temperature of the superfusing saline. In 100% of preparations so far (>35) there has been a tight correlation between the timing of hyperthermic failure of circuit function and an abrupt and dramatic increase in the extracellular K^+ concentration (Fig. 4). When the heater was turned off the saline returned to room temperature whereas the extracellular K^+ concentration remained elevated for variable periods before gradually returning to pre-treatment values. There was an equally tight correlation between
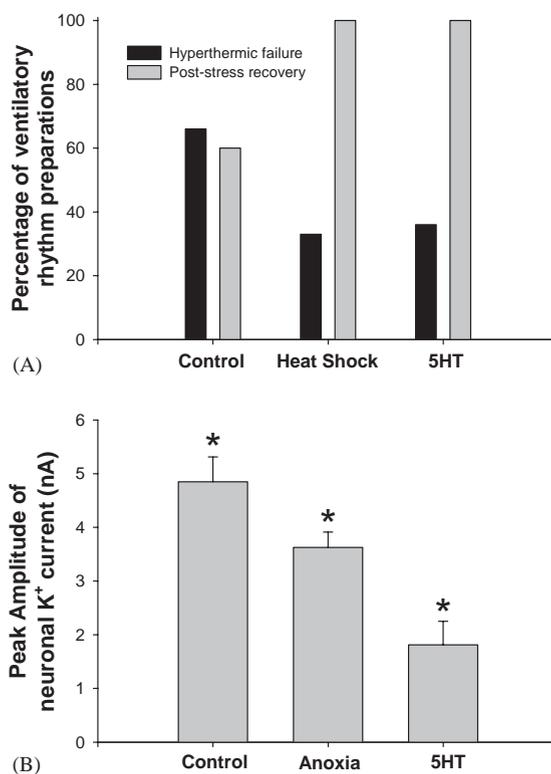

Fig. 3. Serotonin (5HT) mimics the effects of stress pretreatment on neuronal potassium currents and on thermotolerance of ventilatory motor pattern generation in locusts. A. Prior HS reduces the percentage of locust preparations in which operation of the ventilatory central pattern generator fails when held at 45 °C for 20 min (hyperthermic failure) and increases to 100% the percentage recovery of those that failed (post-stress recovery). This effect is mimicked in NHS preparations by superfusion of the thoracic ganglia with saline containing 1 mM 5HT. B. Peak potassium currents in response to a step change to 40 mV from a holding potential of −60 mV recorded by whole cell patch clamp of neuronal somata are reduced by prior anoxia and by superfusion of 200 μM 5HT in the saline (asterisks indicate that each bar is significantly different from the other two). (A modified from Newman et al., 2003; B modified from Lee, 2001).

the recovery of circuit function and the restoration of extracellular K^+ concentration (Fig. 4). Preliminary results indicate that the rate of rise of extracellular K^+ at failure was slower in HS preparations than in controls and that the rate of restoration was faster (C.I. Rodgers and R.M. Robertson, unpublished). Given that one of our most consistent findings is that HS increases time to failure and reduces time to recover, the current results suggest that HS reduces extracellular K^+ build-up (e.g. by down-regulating K^+ conductance as in Fig. 2). They also suggest that HS potentiates the mechanisms that restore the K^+ gradient perhaps by activating the
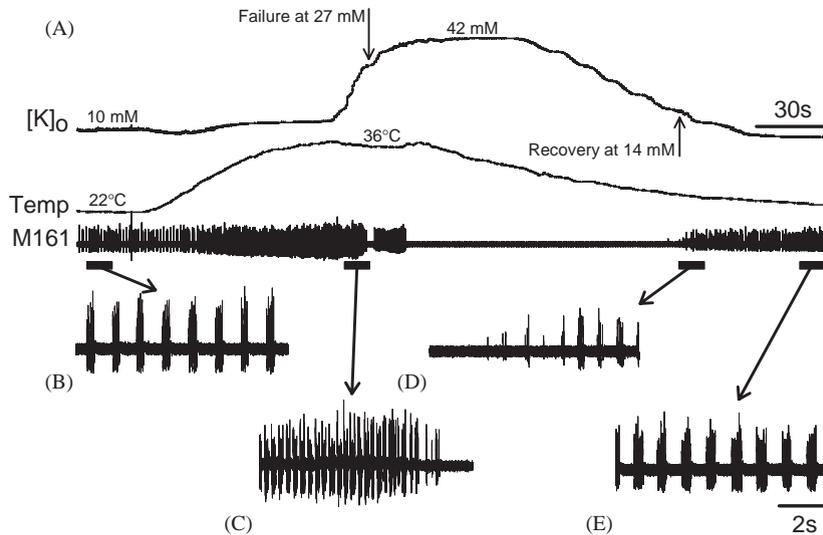
Fig. 4. Timing of hyperthermic failure and post-stress recovery of the locust ventilatory central pattern generator correlates with timing of build-up and restoration of extracellular K^+ concentration. A. Simultaneous recording of extracellular K^+ concentration ($[K]_o$) using a K^+-sensitive microelectrode, temperature of the superfusing saline (Temp) measured at the metathoracic ganglion, and ventilatory motor pattern recorded extracellularly from muscle 161 in the second abdominal segment (M161). B, C, D and E show expansions of the motor pattern trace at selected time points to show more clearly the rhythmical bursting activity. C shows the time of hyperthermic failure of patterning (the activity seen immediately after this in A is non-patterned high-frequency firing of the motoneuron associated with failure). D shows the time of post-stress recovery of the motor pattern. The $[K]_o$ trace is the voltage of the ion-sensitive electrode and is logarithmically related to the K^+ concentration. At the beginning of this sequence the temperature was 22 °C, extracellular K^+ was 10 mM and the ventilatory motor pattern cycled at around 1 Hz. During a temperature ramp to 36 °C the motor pattern frequency increased to around 3 Hz but the extracellular K^+ was stable. Failure of the motor pattern was associated with an abrupt loss in the stability of extracellular K^+ which increased to a plateau of 42 mM. At failure the heater was turned off and the saline temperature returned to 22 °C. Extracellular K^+ remained high for about 30 s before gradually returning to 10 mM. The motor pattern returned after about 2 min of failure and quickly reestablished a robust rhythm at 1 Hz. Motor pattern failure coincided with an extracellular K^+ concentration of 27 mM and recovery coincided with 14 mM. (From C.I. Rodgers and R.M. Robertson, unpublished.)

Na^+/K^+ ATP-ase (sodium pump), which would also slow the temperature-induced build-up. Thus HS and other stresses could activate pathways that target the sodium pump indicating this as a fruitful avenue to pursue for uncovering mechanisms that protect CNS function from hyperthermic failure.

4. Conclusions

Our research suggests that the CNS possesses several mechanisms that can extend the temperature range of operation of neural circuits. Ion channel modulation likely underlies the HS-mediated changes of Q_{10}-like thermosensitivity, whereas HSP-mediated chaperoning may offset more time-dependent thermosensitivities. HS protects locomotor behaviour and circuit function in *Drosophila* which means that the molecular underpinnings of such protection can now be addressed using the powerful molecular genetic approaches enabled by this model system.

On the other hand, the locust model provides an opportunity to link physiological disturbance and protection with an ability to investigate neural circuits using the precision enabled by working with an invertebrate. The ionic disturbance associated with the failure of the ventilatory CPG in locusts is very similar to disturbances associated with spreading depression in mammalian cortex (Somjen, 2001, 2002). Spreading depression can be induced by hyperthermia and it has been suggested that this results from a failure of the sodium pump which could cause febrile seizures (Wu and Fisher, 2000). In other tissues Na^+/K^+-ATPase activity can be made thermotolerant by heat pretreatments (Anderson and Hahn, 1985; Burdon et al., 1984) and this can be associated with HSP-mediated stabilization of the cytoskeleton to prevent the dissociation of the Na^+/K^+-ATPase from the membrane (Bidmon et al. 2002; Vicencio et al., 2003). Chaperones, like HSP70, exert protective functions by refolding denatured proteins or otherwise preventing the build-up of dangerous protein aggregates. Some of the key targets of chaperones are the proteins of the cytoskeleton, which

are both vulnerable to hyperthermia and closely involved in stabilizing and regulating the membrane proteins underlying neural function (ion channels, receptors and the sodium pump) (e.g. Trotta et al., 2004). Thus one of the important roles of HSPs (notably the small HSPs) may be to stabilize the cytoskeleton (Lavoie et al., 1993; An et al., 2004).

In the context of all the above observations it is interesting that the cytoskeleton has been implicated in HS-mediated protection of locust neuromuscular junctions (Klose et al., 2004a). Locusts are well-adapted to hot environments with a robust HS response (Qin et al., 2003), and they have a nervous system that has proven eminently accessible to modern electrophysiological investigation (e.g. Robertson, 1989, 2003); much is known about the neural circuits underlying the critical behaviours of escape jumping and ventilation. With these model systems it will be possible to test the proposition that HS protects CNS operation from hyperthermia via the action of HSPs to stabilize the cytoskeleton and ensure continued operation of important membrane proteins such as ion channels and the Na^+/K^+-ATPase.

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