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Modulation of Neural Circuit Operation by Prior Environmental Stress[1]

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SYNOPSIS. Many organisms are exposed to harsh environmental conditions that may impair the operation of vital neuronal circuits and imperil the animal before these conditions directly cause cell and tissue death. Prior exposure to extreme but sub-lethal stress has long-term effects on neural circuit function enabling motor pattern generators to operate under previously non-permissive conditions. Using several model systems we have been investigating the mechanisms underlying stress-mediated neuroprotection, particularly thermotolerance imparted by a prior heat shock. Prior anoxia and cold shock also impart thermotolerance of motor pattern generation suggesting that different stressors activate common protective pathways. Synaptic transmission, action potential generation and neuronal potassium conductance are modulated by prior heat shock. Pharmacological block of potassium channels, which increases the duration of action potentials and the amplitude of postsynaptic potentials, mimics the thermoprotective effect of a prior heat shock. A universal consequence of heat shock and other stresses is the increased expression of a suite of heat shock proteins of which HSP70 is most closely linked to organismal thermotolerance. Increased levels of HSP70 are sufficient, but not necessary for synaptic thermoprotection. Accumulating evidence suggests the existence of multiple, overlapping pathways for protection and that these mechanisms may be neuron specific depending on their functional roles.

INTRODUCTION

Animal behavior is critically dependent on adequate function of neuronal circuits in the central nervous system (CNS). Monitoring the environment, choosing behavioral strategies and executing appropriate motor acts are all undertaken using patterns of electrical activity generated within the CNS. Much of an animal's physiology is concerned with maintaining the physical environment of circuits in the CNS and when these homeostatic mechanisms go awry, or are inadequate to compensate for an external disturbance, circuit dysfunction and disrupted behaviors ensue. It is important to note that even mild, or predicted, alterations in environmental conditions such as those associated with a changing diet (Xia *et al.*, 1997) or seasonal variations (Rosenthall and Bezanilla, 2000) could be sufficient to exert long-term modulatory effects on neural operation. Much of the variability in behavioral results obtained from different laboratories working with genetically identical strains of mice has been ascribed to idiosyncratic laboratory environments (Crabbe *et al.*, 1999). Indeed, with reference to behavioral testing of genetically engineered mice, ''laboratory environments probably can never be made sufficiently similar to guarantee identical results on a wide range of tests in a wide range of labs'' (Wahlsten *et al.*, 2003). These findings have important neuroecological implications in that behavior and, by extension, neural circuit operation are constrained and modulated by prior ecological experience. In this review I focus on those changes wrought by prior exposure to stressful environments, particularly the changes that can be interpreted as adaptive modifications to allow, or improve, circuit function during a subsequent exposure to harsh conditions.

A particularly potent stressor for neural pattern generation is temperature and dangerous increases in body temperature have numerous pathological sequelae. At the extreme, high temperatures result in cellular death as membranes melt and proteins denature. Prior to this end-point, however, hyperthermia causes the central circuits responsible for proper behaviour to malfunction, impairing health and endangering life. It could be argued that impaired circuit function is more likely to result in organismal death in a harsh environment than accumulating cell death in organ systems. Thus disrupted ventilatory motor patterns or inefficient predator escape mechanisms may result in organismal death long before the thermal dose is sufficient to kill cells. In this context, therefore, it is clearly adaptive for mechanisms to exist that would protect neural circuit operation under environmental stress.

The heat shock (HS) response is a highly conserved cellular response to a variety of different stressors including ischaemia, free radicals, excitatory amino acids and high temperature (Morimoto and Santoro, 1998; Sharp *et al.*, 1999). The response is characterized by a rapid transcriptional activation of genes (Morimoto, 1993) resulting in increased levels of several heat shock proteins that are distinguished according to their molecular weights (*e.g.*, HSP40, HSP60, HSP70, HSP90). HSPs act as molecular chaperones to assist in refolding proteins to their native states or as proteases to break down denatured protein aggregates (Feder and Hofmann, 1999). Their roles in mediating acquired thermotolerance and modifying physiological stress responses are becoming clearer (Kregel, 2002) and up-regulation of HSP70 levels using transgenic mice (Plumier *et al.*, 1997; Rajdev *et al.*, 2000) and

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virally-mediated gene transfections (Yenari *et al.*, 1998) has been shown to reduce the neural damage in experimental models of stroke. There is, however, very little information available about how HSPs might protect circuit function as opposed to reducing the extent of neuronal death. In fact little is known about the mechanisms underlying stress-mediated long-term protection of neuronal circuitry, whether HSPs are involved or not. Insect model systems lend themselves particularly well to neuroethological and neuroecological studies and for several years my collaborators and I have been investigating such circuit protection, primarily in the migratory locust, but more recently using larval *Drosophila*.

STRESS-MEDIATED MODULATION OF MOTOR CIRCUITS AND BEHAVIOR

Locust ventilation

In quiescent grasshoppers the ventilatory motor pattern that drives abdominal pumping is intermittent (Harrison, 1997). Often the first behavioural response to a stimulus is a change in the nature of ventilation (Hustert, 1975) and alert animals exhibit a continuous abdominal pumping. It is well established that the ventilatory motor pattern is produced by a central pattern generator (Miller, 1966) and an isolated nervous system is able to produce both the motor rhythm and a pattern of ventilation characterized by interspersed bouts of abdominal pumping and miniature ventilations (Bustami and Hustert, 2000). The rate of abdominal pumping increases with increasing temperature (Miller, 1966) and this is likely an adaptive response to promote evaporative heat loss (Prange, 1990). It has long been suggested that insects close the spiracles to prevent desiccation, minimizing water loss but exposing themselves to hypoxic stress (Lighton, 1996). Increasingly this idea is challenged (*e.g.*, Rourke, 2000) but there is little doubt that temperature stress, hypoxic stress and desiccation stress are tightly linked. These characteristics make the locust ventilatory circuitry ideal for investigations of stress-mediated protective modifications to neuronal function. We have recently started to investigate stress tolerance in this system (Newman *et al.*, 2003) and our preliminary results serve well to illustrate the concepts of thermal dose and cross-tolerance.

An acute temperature ramp, increasing at around 6°C/minute from room temperature until the ventilatory motor pattern is observed to fail, causes the frequency of abdominal pumping to increase from 1 cycle/s to around 3 cycles/s at 50°C. However for this system, unlike others described below, the temperature recorded at failure is not affected by pre-exposure to stressful conditions. Nevertheless it is a robust finding for all the systems we have examined that the proportion of preparations recovering, and the time taken to recover, are strongly influenced by a prior stress, with control animals being less likely to recover and taking longer to do so. This effect on recovery from failure

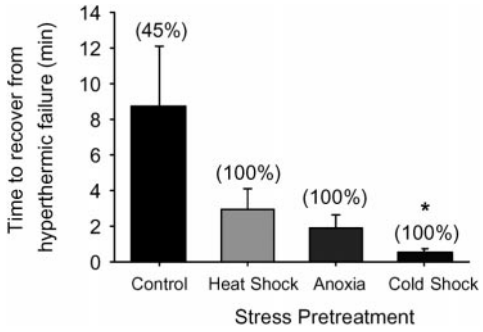

FIG. 1. Stress-induced thermotolerance of the ventilatory circuit in locusts. After heat-induced failure, control preparations take longer to recover than preparations taken from locusts that have experienced prior heat shock (3 hr at 45°C), anoxia (2 hr in 100% N_2) or cold shock (3 hr at 3°C). Note that fewer than 45% of control preparations recover whereas 100% of preparations from pre-stressed locusts recover. Treatment has a significant effect on time to recover (Kruskal Wallis, $P < 0.05$); asterisk indicates significant difference from control in multiple pair-wise comparisons. Modified from Newman *et al.* (2003).

is similar for three different types of environmental stress: heat shock, anoxic coma, and cold shock (Fig. 1). This demonstrates cross-tolerance such as has been described for thermotolerance of action potential generation in flight system motoneurons (Wu *et al.*, 2002). It is important to note that in the latter study cross-tolerance appears unidirectional with prior anoxia inducing thermotolerance but heat shock being unable to protect against some consequences of anoxia. The results also suggest that any repair process is time-dependent, similar to the time-dependence of thermal damage. The intensity of a heat stress is determined by the absolute temperature, the rate of heating and the length of time at damaging temperatures *i.e.*, the thermal dose. Maintaining the ventilatory circuit at around 45°C causes control animals to fail after 15 to 20 min. whereas heat shocked animals continue to generate ventilatory motor patterns for more than 30 min. at this temperature. The mechanisms underlying thermoprotection in the ventilatory system remain to be determined but it may prove easier to discover them in a system that normally is continually active and thus whose failure indicates failure of the circuit rather than a lack of sufficient motivation or sensory drive.

Locust flight

Locust flight is a rhythmical locomotor behaviour that has received considerable research attention for more than half a century. Although the mechanisms for central pattern generation in this system are still incompletely understood there is information available about the central circuits (*e.g.*, Robertson, 1989) and it is a useful model system for investigating the role of proprioceptive input and the constraints on circuit function (*e.g.*, Robertson, 2003). One constraint on locust flight behaviour is ambient temperature. To a large extent the flight circuit operates at temperatures determined by ambient temperature plus the heat generated

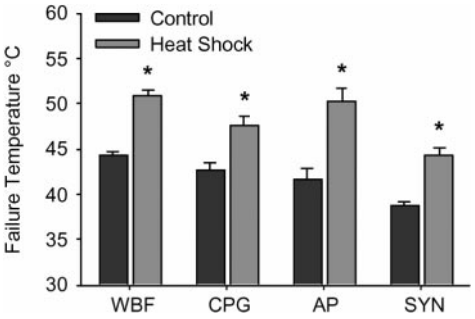

FIG. 2. Heat-shock induced thermotolerance in the locust flight system. Control preparations fail at higher temperatures than preparations taken from locusts that have experienced a prior heat shock (3 hr at 45°C). This is observed for wingbeat frequency of intact tethered locusts (WBF), rhythm frequency of the flight central pattern generator (CPG), action potential generation of wing muscle motoneurons (AP) and synaptic potentials recorded in flight interneurons and motoneurons after action potentials of the forewing hinge stretch receptor (SYN). Asterisks indicate significant difference from control (Student's *t*-test, $P < 0.05$). Modified from Robertson *et al.* (1996), Wu *et al.* (2001) and Dawson-Scully and Robertson (1998).

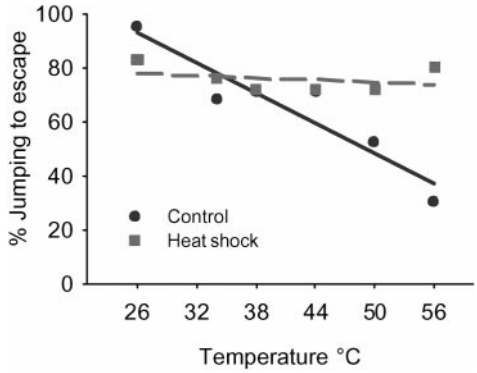

FIG. 3. Heat shock-induced thermoprotection of jumping escape behaviour in the locust. Control animals are less likely to jump to avoid capture at high ambient temperatures than animals that had experienced a prior heat shock (3 hr at 45°C). Jumping is not contingent on temperature in heat shock animals (Chi square, $P > 0.9$) but is contingent on temperature in control animals (Chi square, $P < 0.01$). Modified from Barclay and Robertson (2000).

by working flight muscles, with accepted limits for normal flight activity at 24°C (permissive for flight initiation, Weis-Fogh 1956) and 42°C (failure of flight muscle contraction, Neville and Weis-Fogh, 1963). Within this range there is an effect of temperature on wingbeat frequency (0.3 Hz increase per °C) that can be attributed to action on the central pattern generator (Foster and Robertson, 1992). Postsynaptic potentials (PSPs) in the system have maximum amplitude at around room temperature (~24°C), diminishing with both lower and higher temperatures (Robertson, 1993). The balance of excitation and inhibition in the circuitry affords some measure of automatic compensation for temperature changes. Systemic reduction of excitatory and inhibitory PSP amplitude using zero calcium in the superfusing saline has remarkably little effect on the centrally generated motor pattern frequency; but upsetting the balance by reducing inhibitory PSPs selectively using the Cl^- channel blocker picrotoxin renders the central circuit more thermosensitive (Xu and Robertson, 1996). This suggests that PSP amplitude sets permissive limits for operation of the circuit but that the frequency of the output is determined by temporal parameters of synaptic and axonal conduction delay.

There is little doubt that locusts in their natural habitat are exposed to daily cycles of ambient temperature that can reach extremely high levels. The demonstration of induced thermotolerance and a cellular heat shock response in locusts (Whyard *et al.*, 1986) emphasizes the notion that locusts are well adapted to this habitat. The adaptation extends to the level of neural circuit operation and it was an important finding that the locust flight system can be conditioned by prior heat experience to permit operation in a higher temperature range (Robertson *et al.*, 1996). In particular the wing beat frequency of intact tethered locusts flying in a wind stream becomes essentially insensitive to temperature increases (0.04 Hz per °C) after a prior heat shock. Also the temperature at which the system fails, indicated by an inability for locusts to sustain wingbeating, increases from 44°C to 51°C after heat shock. This is mirrored at the level of central pattern generator output for which the failure temperature increases from 43°C to 48°C after heat shock. Indeed the increase in failure temperature at all levels of the system from behavioural output to signal generation is a robust finding in the flight system of the locust (Fig. 2).

Locust predator avoidance

Attempting to catch a grasshopper on a hot summer day is a well-known and frustrating experience for many children, ducks, and researchers, as the intended victim detects the approaching predator and activates a powerful jumping circuit. I suggested above that predator avoidance would be a behaviour important to protect for high temperature operation. There is evidence that previous heat experience can change the behavioural strategy employed by locusts in attempting to evade capture. Control animals become less likely to jump away at such times as temperature increases, whereas animals that have been heat shocked maintain their ability to jump to escape (Barclay and Robertson, 2000; Fig. 3). This may indicate a behavioural choice or it may simply indicate that the neural links from detection to execution are protected at high temperature in heat-shocked animals. At one end of this reflex, effective escape depends on the rapid extension of the femoral-tibial joint of the hindlegs using the powerful Extensor Tibiae (ETi) muscle. Prior heat shock modulates operation of the neuromuscular junction of the fast motoneuron to this muscle (FETi) making this an important model for investigating mechanisms of synaptic thermotolerance (Barclay and Robertson, 2000, 2001, see also Klose and Robertson, 2004). At the other end, effective escape depends on the ability to detect rapidly looming stimuli and faith-

fully relay this information to the locomotor circuits in the thoracic ganglia.

The descending contralateral movement detector (DCMD) neuron is a visual interneuron that receives strong input from the lobula giant movement detector (LGMD) that responds with high frequency firing to looming visual stimuli (Rowell, 1971; Rind and Simmons, 1992; Gabbiani *et al.*, 1999). LGMD/DCMD has properties better suited for predator avoidance than collision avoidance during flight (Gray *et al.*, 2001). The accepted understanding is that the synaptic connection from LGMD to DCMD, though chemical, is strong enough to ensure that DCMD fires in a 1:1 fashion with LGMD action potentials (Rind, 1984). The behavioural importance of this relay suggests that it will be protected by heat shock. Preliminary results confirm this and show in addition that the response to looming stimuli is potentiated by a prior heat shock (Anstey *et al.*, 2003). Thus the conditioning has not simply allowed the circuitry to continue to operate but has changed the nature of the response, showing true environment-dependent phenotypic plasticity.

EFFECTS OF PRIOR STRESS ON NEURAL SIGNALS

Synaptic potentials

The connections from the forewing hinge stretch receptor (fSR) to interneurons and motoneurons of the flight systems (Reye and Pearson, 1987) are useful models for investigating the modulatory effects of temperature on synaptic function (Robertson, 1993). Prior heat shock has two profound effects on the temperature sensitivity of synaptic parameters in this system (Dawson-Scully and Robertson, 1998). First, it reduces the loss of amplitude that is evident with increasing temperatures. If a threshold PSP amplitude is permissive for circuit function (see above) then this effect of heat shock can explain the extension of the upper temperature limit for operation of both the flight system as a whole and the central pattern generator, *i.e.*, the reduction to a threshold percentage of the original PSP amplitude occurs at around 5°C higher temperatures after heat shock. Second, heat shock has potent effects on synaptic delay. In the range of temperatures for normal operation of the flight system (25°C to 45°C) synaptic delay decays exponentially in control preparations but is constant in heat shock preparations. This observation can account for the temperature insensitivity of wingbeat frequency seen in heat shocked locusts. Further research on the modulatory effects of heat shock on synaptic operation has been pursued with the more accessible neuromuscular junction of locust FETi and of *Drosophila* larval abdominal muscles (Klose and Robertson, 2004). It is important to point out that whereas this work is being undertaken with insect preparations, similar protective effects of prior heat shock on synaptic function are also evident in mammalian preparations (Kelty *et al.*, 2002). The respiratory circuit in the brain stem of mice is vulnerable to hyperthermia (Tryba and Ramirez, 2003). Indeed hyperthermic failure of respiratory pattern generation is a likely cause of infant death and it has been suggested that an impaired heat shock response may be causative in SIDS (Gozal, 1996). Whole cell recordings from respiratory neurons in the pre-Boetzinger nucleus reveal that the barrage of miniature postsynaptic currents evident during hyperthermia is prevented by a prior heat shock treatment of the brain slice (Kelty *et al.*, 2002).

Action potentials

Action potentials are critical for circuit function by relaying information over long distances and by triggering the influx of Ca^{++} that is necessary for transmitter release at synapses. The effects of temperature on the properties of action potentials are well known and predictable from the Hodgkin-Huxley equations. Popular neural simulation programs such as Neurosim (Revest, 1995) demonstrate the decrease in amplitude and duration and ultimate spike failure as temperature increases. This occurs as a result of K^+ currents activating more rapidly and overwhelming the Na^+ current before the latter has a chance to develop fully. It is possible in these simulations to rescue the action potential by reducing the magnitude of the K^+ conductance. It was an early observation that the temperature sensitivity of action potentials recorded in different species is different and appropriate for the different thermal environments of the species (Hodgkin and Katz, 1949). More recently it has been demonstrated that squids inhabiting different environments have action potentials with different durations when measured at the same temperature (long duration for warm water species and short duration for cold water species; Rosenthall and Bezanilla, 2002). They also have different failure temperatures (higher for longer duration action potentials). The difference results from variation in the magnitude of Na^+ and K^+ conductances and it is suggested that the differences in duration are adaptive primarily to avoid action potential failure at high temperatures in the squids from warmer habitats. Reduced K^+ conductance competes less with the Na^+ conductance allowing the action potential to develop. Given this evolutionary adaptation for higher temperature operation one might predict that short-term modulation for high temperature operation would similarly involve a reduction of K^+ currents and increase in action potential duration.

The action potential of the fSR can be unambiguously identified in extracellular recordings of nerve root activity because of its size and characteristic response to forewing elevation. The triphasic action potential in monopolar extracellular recordings is an accurate reflection of the intracellular action potential (Pearson *et al.*, 1970). A preliminary investigation of the effects of heat shock on axonal conduction by measuring parameters of the extracellular fSR action potential revealed that the amplitude was rendered less thermosensitive but that there was no significant difference in the duration (Gray and Robertson, 1998).

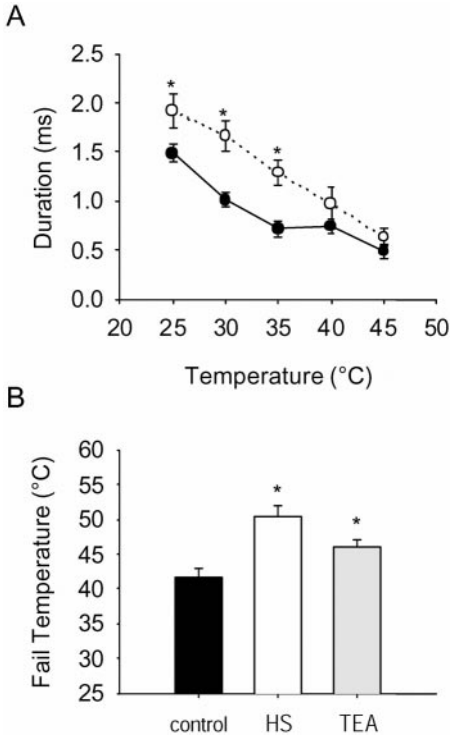

FIG. 4. Thermoprotection of action potentials in flight motoneurons. A. Prior heat shock (open circles) increases the duration of action potentials measured at half amplitude compared with those recorded from control animals (closed circles). Asterisks indicate significant differences from controls (two-way repeated measures ANOVA and Tukey multiple pairwise comparisons). B. Blockade of K^+ currents with tetraethyl ammonium (TEA) mimics the protective effect of heat shock (HS) by increasing the failure temperature relative to controls (C). HS and TEA are not different from each other. Asterisks indicate significant difference from controls (one-way ANOVA and Dunnet's multiple pairwise comparisons). Modified from Wu *et al.* (2001).

Action potentials recorded intracellularly from the neuropile processes of wing muscle motoneurons do however demonstrate a profound increase in duration measured at half amplitude (1.5 ms to 2 ms) (Wu *et al.*, 2001; Fig. 4A). A potential confound in the latter investigation is that action potentials were recorded in passive membrane, distant from the site of generation, and thus had been filtered by cable properties. It is still unclear whether motoneuronal cable properties are affected by the prior heat shock but the fact that the reduction of duration is independent of changes in amplitude suggests that the results are not simply due to altered filtering properties of the passive dendritic membrane. Moreover whole cell patch clamp recordings of neuronal somata in a metathoracic ganglion slice demonstrate that K^+ conductance is reduced both by prior heat shock (Ramirez *et al.*, 1999) and by prior anoxia (Wu *et al.*, 2002), consistent with an increase in action potential duration. Finally, pharmacological reduction of K^+ conductance using tetraethyl ammonium both increased the duration of action potentials in motoneurons and increased the temperature at which they failed (Wu *et al.*, 2001; Fig. 4B).

Recently we have tried to resolve this issue by making intracellular recordings from the axon of the DCMD as it descends in the meso-metathoracic connective. This approach avoids the limitations associated with extracellular recording and intracellular recording at sites electrically distant from the events of interest. Preliminary results indicate that amplitude and duration of the DCMD action potential are not significantly altered by heat shock, though there are significant changes in the excitability of the axonal membrane that could account for the induced thermotolerance (Money *et al.*, 2003).

Reconciliation of these disparate results is possible by considering the functional roles of the different neurons. Flight motoneurons often fire only a single action potential in each cycle of the motor pattern and the frequency of doublet firing is around 70 Hz. In contrast to this, both the fSR and the DCMD are neurons that normally fire at high frequencies. Mature adults at room temperature show fSR firing around 300 Hz in response to wing elevations (Gray and Robertson, 1994), and fSR recordings using implanted electrodes in intact animals during tethered flight reveal frequencies of 500 Hz (Möhl, 1979). At room temperature DCMD responds to optimal looming stimuli with an accelerating discharge that reaches around 300 Hz (Gray *et al.*, 2001; Gabbiani *et al.*, 1999). After heat shock and at high temperature (around 40°C) the same stimulus can evoke a discharge up to 1000 Hz in DCMD (Anstey *et al.*, 2003). A protective mechanism that increases the duration of action potentials would prevent firing at high frequencies and compromise the functional role of these neurons. Thus it is likely that different mechanisms of thermoprotection can be induced in neurons by heat shock depending on their normal activity patterns and that there is unlikely to be a single strategy that would be suitable for all.

MECHANISMS OF STRESS-MEDIATED PROTECTION

The role of the heat shock response

Much interest in many different organisms has been directed towards the role of HSP70 in thermotolerance and this is an obvious candidate for mediating the protection of neural circuits after stress. Locusts have a heat shock response (Whyard *et al.*, 1986) and recently we have cloned HSP70 in this organism (Qin *et al.*, 2003). It appears that HSP70 is constitutively expressed in *L. migratoria* and the effects of heat shock on levels of protein and transcript are relatively mild. This can be interpreted as a fit to this organism's relatively harsh natural habitat but raises the question of whether the response is sufficient to mediate the observed protection in our experiments. At the larval neuromuscular junction in *Drosophila* there is strong evidence that HSP70 is involved in synaptic protection (Karunanithi *et al.*, 1999, 2002; see also Klose and Robertson, 2004). In the mouse brain, however, HSP70 is sufficient, but not necessary, for stabilizing

synaptic function at hyperthermic temperatures (Kelty *et al.*, 2002). The nature and extent of HSP70s role in mediating thermoprotection at the various neural levels described above are still unclear. Moreover it is quite likely that the other HSPs could have significant or more potent roles, according to their sites of action. Finally, there are good reasons for thinking that parallel, and perhaps more rapid, mechanisms could involve neuromodulators, such as serotonin, activating signalling pathways that terminate with phosphorylation of membrane proteins such as ion channels and receptors. Indeed it would be surprising if there were not multiple, overlapping protective pathways. The challenge now is to unravel these interlaced pathways.

Molecular genetic approaches

Whether HSPs are involved or not it is quite obvious that the most powerful approaches for uncovering the cellular and subcellular mechanisms of stress-mediated protection of neural operation will be those using genomic model systems. *Drosophila* provides one such model system and the techniques to monitor genome responses to different treatments of the animal (see Karunanithi, ??), and to manipulate gene expression with precise temporal and spatial resolution, are well established and powerful. As mentioned above for investigation of the role of HSP70 at the neuromuscular junction, we have started taking molecular genetic approaches. The next step is to investigate CNS operation in this model organism and we have developed a preparation of the larva that will produce centrally generated locomotor patterns (Barclay *et al.*, 2002). Prior heat shock increases the upper temperature limit for peristaltic locomotion of intact larvae and there is a parallel protection of the locomotor pattern generator (Chu *et al.*, 2003). The future with this preparation holds rich possibilities for dissecting the mechanisms that protect neural circuit function, and not merely neuronal survival, in the face of extreme environmental conditions.

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