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Na^+-K^+-ATPase trafficking induced by heat shock pretreatment correlates with increased resistance to anoxia in locusts

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Hou N, Armstrong GA, Chakraborty-Chatterjee M, Sokolowski MB, Robertson RM. Na^+-K^+-ATPase trafficking induced by heat shock pretreatment correlates with increased resistance to anoxia in locusts. *J Neurophysiol* 112: 814–823, 2014. First published May 21, 2014; doi:10.1152/jn.00201.2014.—The sensitivity of insect nervous systems to anoxia can be modulated genetically and pharmacologically, but the cellular mechanisms responsible are poorly understood. We examined the effect of a heat shock pretreatment (HS) on the sensitivity of the locust (*Locusta migratoria*) nervous system to anoxia induced by water immersion. Prior HS made locusts more resistant to anoxia by increasing the time taken to enter a coma and by reducing the time taken to recover the ability to stand. Anoxic comas were accompanied by surges of extracellular potassium ions in the neuropile of the metathoracic ganglion, and HS reduced the time taken for clearance of excess extracellular potassium ions. This could not be attributed to a decrease in the activity of protein kinase G, which was increased by HS. In homogenates of the metathoracic ganglion, HS had only a mild effect on the activity of Na^+-K^+-ATPase. However, we demonstrated that HS caused a threefold increase in the immunofluorescent localization of the α-subunit of Na^+-K^+-ATPase in metathoracic neuronal plasma membranes relative to background labeling of the nucleus. We conclude that HS induced trafficking of Na^+-K^+-ATPase into neuronal plasma membranes and suggest that this was at least partially responsible for the increased resistance to anoxia and the increased rate of recovery of neural function after a disturbance of K^+ homeostasis.

Locusta migratoria; insect; suffocation; anoxia; heat shock; extracellular potassium; sodium pump; immunohistochemistry; PKG

AN ANIMAL'S ABILITY TO WITHSTAND periods of anoxia is critically dependent on the sensitivity of its nervous system to an interrupted energy supply. This sensitivity can be modulated by acute pretreatments or pharmacological or genetic manipulations (Dawson-Scully et al. 2010), demonstrating the existence of intrinsic signaling pathways that could activate mechanisms for such phenotypic plasticity. There is still much to learn about these mechanisms, particularly in organisms like insects that are robustly hypoxia-tolerant (Harrison et al. 2006; Hoback and Stanley 2001; Zhao et al. 2013), and here we investigated the effects of a heat shock pretreatment (HS) on the sensitivity to suffocation by immersion in the locust, *Locusta migratoria*.

Due to their small size and cryptic habitats, insects are often exposed to periodic flooding (Brust et al. 2007; Woodman 2013) and have adapted differential survival capabilities (Brust and Hoback 2009; Brust et al. 2005; Plum 2005). *L. migratoria* are adapted to a natural habitat in semi-arid regions of Africa, Asia and Oceania, where they are regularly exposed to variable and extreme temperatures and oxygen partial pressure (e.g., low oxygen on the Tibetan plateau). They are also exposed to anoxia as a result of suffocation during the heavy and widespread rains that provide the optimal breeding conditions (Hemming et al. 1979; Muller 1976; Rainey 1951). The effects of the harsh environmental transitions are intricately associated with development of individuals (Rainey 1951), from a near-aquatic egg to the developmental plasticity that accounts for population transformations from solitarious hoppers to gregarious locusts (Pedgley 1979; Tian et al. 2011; Zhang et al. 2009). As a result of their evolution under these harsh conditions, adult locusts can survive extreme heat (>50°C) and several hours of anoxia.

Ventilation is responsible for effective gas exchange throughout the animal and is controlled by neurons in the metathoracic ganglion. The ventilatory central pattern generator (vCPG) is particularly robust, operating even when experimentally isolated (Bustami and Hustert 2000). Maintenance of vCPG activity is particularly important during heat stress because the increased ventilatory rate dissipates body heat (Rodgers et al. 2006). A relaxed locust shows discontinuous ventilation, but when stressed ventilation is continuous. This makes it easy to determine when the system has arrested and when it has recovered. Thus it provides an ideal system for studying vulnerability and protection of neuronal circuits (Newman et al. 2003).

At extremes of temperature, in the absence of oxygen, or during treatments that impair mitochondrial operation, locusts enter a reversible coma, which is associated with a surge in extracellular potassium ion concentration ($[K^+]_o$) in the nervous system that prevents neural function (Money et al. 2009; Rodgers et al. 2007, 2010). HS (45°C, 3 h) increases the temperature at which neural function arrests during hyperthermia and speeds the recovery on return to normal temperatures (Dawson-Scully and Robertson 1998; Robertson 2004a, 2004b; Robertson et al. 1996; Wu et al. 2001). The increase in the speed of recovery is associated with an increase in the rate at which excess K^+ ions are cleared from the extracellular space (Rodgers et al. 2007), suggesting a role for the Na^+-K^+-ATPase (Emery et al. 1998). However, increased activity of the Na^+-K^+-ATPase could not be detected in homogenates of the metathoracic ganglion, prompting a suggestion that HS may have affected the trafficking of Na^+-K^+-ATPase complexes in

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neuronal and glial membranes (Rodgers et al. 2007) as is the case for pump regulation in skeletal muscle (Benziane and Chibalin 2008) and in snail neurons exposed to a static magnetic field (Nikoliç et al. 2013).

We investigated the effect of a HS on the sensitivity of the locust nervous system to anoxia induced by immersion under water. We showed in dissected preparations that an immersion-induced coma is accompanied by a surge in $[K^+]_o$ and that prior HS speeds the recovery of ventilatory motor activity from chemical anoxia induced by sodium azide and from similar surges induced by injections of KCl into the neuropile. To determine whether the anoxia tolerance we found could be attributed to decreased activity of protein kinase G (PKG), as previously demonstrated (Dawson-Scully et al. 2010), we assayed PKG activity and found, in contrast, that it increased in thoracic ganglia after HS. Finally, we showed that HS had no obvious effect on the abundance of Na^+-K^+-ATPase in homogenates of the metathoracic ganglion, but that it had a profound effect on the cellular distribution of the α-subunit of Na^+-K^+-ATPase within metathoracic neurons, indicating a trafficking of Na^+-K^+-ATPase into the plasma membrane. These results are consistent with a model whereby the HS-induced increased rate of $[K^+]_o$ clearance after hyperthermic (Rodgers et al. 2007) and anoxic comas is aided by HS-induced trafficking of Na^+-K^+-ATPase into neuronal plasma membranes.

MATERIALS AND METHODS

Animals. African migratory locusts, *Locusta migratoria migratoriodes*, were raised in a crowded colony maintained in the Biosciences Complex located at Queen's University in Kingston, Ontario. The animals were reared under a 12:12-h dark-light regime with lights on at 0700. Each cage was individually lit with a 40-W incandescent light bulb for light and warmth. Room temperature was maintained at 25 ± 1°C. All animals were provided daily with fresh wheat grass, carrot slices or tomato pieces, and an ad libitum mixture of 1 part skim milk powder, 1 part torula yeast, and 13 parts bran by volume. Adult locusts 4–6 wk past the final moult were taken from the colony and held in the laboratory in ventilated plastic containers, without access to food or water, prior to use. They remained in these containers for the 4 h of pretreatment (control and HS) and recovery and subsequently for variable periods as they were used in experiments. Electrophysiological experiments and tissue collection were performed between 1300 and 1700 to control for possible circadian effects (Gorska-Andrzejak et al. 2009).

Heat shock. Locusts for HS preconditioning were placed in a humid incubation chamber at 45°C for 3 h, followed by recovery for 1 h at room temperature. Humidity was not controlled, but was maintained at a high level by placing in the chamber a 500-ml beaker of water, wicked with a paper towel, and inaccessible to the locusts. Control locusts were held at room temperature for 4 h.

Anoxic coma. We chose to induce anoxic comas by suffocation under water because this method is most ecologically relevant and avoids potential unnatural consequences of using different gases such as nitrogen or carbon dioxide. Locusts within a perforated container were submerged in de-chlorinated tap water at room temperature for 30 min. We measured the time taken to enter an anoxic coma characterized by leg kicking and convulsions followed by immobility. At the end of the anoxic treatment, locusts were removed from the water and placed on a paper towel. All animals recovered without apparent harm (walking, jumping, and eating normally), and we measured the times taken for ventilatory movements of the abdomen to resume and then for the locusts to stand with their characteristic posture (i.e., with weight distributed among the legs and the thorax held off the substrate).

Electromyography. Locusts were pinned to a cork board with their wings and legs removed. The thoracic ganglia were exposed via a dorsal dissection (Robertson and Pearson 1982) and stabilized on a stainless steel plate. The preparation was superfused with a continuous flow of saline (147 mM NaCl, 10 mM KCl, 4 mM $CaCl_2$, 3 mM NaOH, 10 mM HEPES dissolved in distilled water, pH 7.2). Electromyograms were recorded by placing a copper wire EMG electrode on ventilatory muscle 161. The copper wire (tip area, 0.32 mm^2) was insulated with a polypropylene coating, except at the tip. Ventilatory motor activity was amplified (Grass P15 AC Preamplifier), digitized using a MiniDigi A1 Two-Channel Acquisition System (Axon Instruments), and saved onto a computer using AxoScope 9.0 (Molecular Devices). To induce chemical anoxia, the saline flow was stopped, and 2 ml of 1 mM sodium azide were pipetted into the thoracic cavity. Resuming the saline flow resulted in recovery of ventilatory motor pattern generation.

Extracellular K^+ measurement. Potassium-sensitive microelectrodes were made from unfilamented glass pipettes with a diameter of 1 mm (World Precision Instruments, Sarasota, FL). They were washed with methanol (99.9%, Sigma-Aldrich), dried on a hot plate prior and pulled to a resistance of 4–8 MΩ. Following silanization with hot dichlorodimethylsilane (100°C, 99%, Sigma-Aldrich) vapor, the electrode tips were filled with Potassium Ionophore I-Cocktail B (5% Valinomycin, Sigma-Aldrich) and back-filled with 500 mM KCl. Reference electrodes were made from filamented glass pipettes, not silanized, and back-filled with 3 M KCl. Electrodes were connected to a DUO 773 two-channel intracellular/extracellular amplifier (World Precision Instruments, Sarasota, FL). They were calibrated using 15 mM and 150 mM KCl solutions to determine the voltage change resulting from a 10-fold difference in $[K^+]_o$ (mM). Voltage measurements were converted to $[K^+]_o$ using the Nernst equation (see Rodgers et al. 2007 for details). For $[K^+]_o$ recording while the preparation was immersed, the cork board was fixed in a container that allowed saline to cover the locust's spiracles and subsequently be removed. Surges of $[K^+]_o$ were induced by 50-nl injections of 150 mM KCl into the neuropile of the metathoracic ganglion using pressure injection from a glass micropipette. Some preparations were pretreated prior to KCl injection with 10-min bath application of 10 μM KT5823 (an inhibitor of PKG) or 100 μM T-0156 (a phosphodiesterase inhibitor to increase levels of PKG). These concentrations have been established as effective in this preparation (Armstrong et al. 2009).

PKG assay. PKG enzyme activity assays were performed on meso- and metathoracic ganglia using the procedure described (Lucas et al. 2010). Ganglia were dissected on ice from male locusts (12 control and 12 HS), flash frozen in liquid nitrogen and stored at −80°C. Subsequently, individual ganglia were homogenized on ice in 25 mM Tris (pH 7.4), 1 mM EDTA, 2 mM EGTA, 0.05% Triton X-100, 5 mM β-mercaptoethanol (Sigma Aldrich) with protease inhibitor cocktail (Roche Diagnostics, Laval, QC, Canada). Samples were sonicated four times for 5 s on ice in a Branson Sonifier 250 (Branson) using the lowest setting (duty cycle 20 and output control 4). Samples were centrifuged for 10,000 RPM at 4°C for 5 min. Each sample's supernatant was then removed, and total protein was determined using a Bio-Rad Laboratories protein assay kit. This was done prior to the cGMP-dependent protein kinase activity assays. The final PKG mixture contained 40 mM Tris-HCl (pH 7.4), 20 mM magnesium acetate, 0.2 mM [γ-^{32}P]ATP (500–1,000 counts·min^{-1}·$pmol^{-1}$) (Amersham, Pharmacia Biotech, Baie D'Urfe, QC, Canada), 13 μg/ml of a heptapeptide substrate highly specific to the PKG (RKRSRAE) (Promega) and 3 μM cGMP (Promega, Burlington, ON, Canada) and 4.6 nM of a highly specific cAMP-dependent protein kinase inhibitor (Ki50% = 2.3 nM) (5–24 from Calbiochem). Control reactions were performed in the presence of 468 nM of a PKG inhibitor KT5823 (Calbiochem) and in the absence of cGMP. Reaction mixtures were incubated for 10 min at 30°C. Reactions were terminated by spotting

70 μl of the reaction mixture onto Whatman P-81 filters, which were then soaked with 75 mM H_3PO_4 for 5 min and washed three times with 75 mM H_3PO_4 to remove any unbound [^{32}P]ATP. Filters were rinsed with 100% ethanol and air dried before quantification. Counts were taken in a Wallac 1409 Liquid Scintillation Counter (Perkin Elmer, Woodbridge, ON, Canada) using a universal scintillation cocktail (ICN) for quantification of PKG activity. The specific PKG activity was expressed as picomoles of ^{32}P incorporated into the PKG substrate per minute per milligram of protein.

Na^+-K^+-ATPase assay. Na^+-K^+-ATPase activity was assessed using a pyruvate kinase/lactate dehydrogenase assay in the presence and absence of ouabain. Single metathoracic ganglia were dissected from control and HS locusts, flash frozen and stored at −80°C for subsequent analysis. Later, individual ganglia were homogenized on ice in 300 μl of lysis buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole and 0.1% deoxycholate, pH 7.3). ATPase activity was measured as the production of NADH spectrophotometrically for 10 min at 340 nm. Each sample was assayed in 6 wells on the 96-well plate, 3 containing ouabain. Each well contained a 300-μl reaction mixture consisting of the following solutions: 228 μl of assay buffer (50 mM imidazole, 100 mM NaCl, 20 mM KCl, and 5 mM $MgCl_2$), 3 μl each of 0.2 mM NADH, 0.5 mM PEP, 10 U/ml lactate dehydrogenase, and 10 U/ml pyruvate kinase, 15 μl of either ouabain (0.5 mM) or assay buffer, and 30 μl sample homogenate. The reaction was started with the addition of 15 μl ATP (3 mM). The slope of absorbance over 10 min was compared between samples with and without ouabain to give a value for activity of only Na^+-K^+-ATPase. This value was normalized to total protein within the sample, which was measured from each homogenate using a standard protocol based on the Bradford method (Bradford 1976).

Western blotting for Na^+-K^+-ATPase. The α_5-antibody, specific for the α-subunit of Na^+-K^+-ATPase, and AA4.3 antibody, specific to α-tubulin, were obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). Metathoracic ganglia from six animals (either heat shocked or control) were pooled in 100-μl ice-cold SEI buffer (0.3 M sucrose; 0.02 M EDTA; 0.1 M imidizole pH 7.3) containing protease inhibitors, sonicated and centrifuged (2,000 *g*) for 5 min at 4°C. Supernatants were retained and quantified using the Bio-Rad protein assay. Six micrograms of protein were separated using SDS-PAGE electrophoresis and transferred to Immobilon polyvinylidene difluoride membrane (EMD Millipore, Billercia, MA), according to the manufacturer's instructions. The membrane was incubated with anti-Na^+-K^+-ATPase at 1:1,000 for 12 h and anti-α-tubulin at 1:1,000 for 1 h followed by 1 h in horseradish peroxidase-conjugated goat anti-mouse at 1:1,000. The Western blot was scanned and quantified using Image Pro Plus (Media Cybernetics, Rockville, MD).

Immunohistochemistry. Metathoracic ganglia were dissected from nine control and nine HS locusts and fixed in 4% formaldehyde at room temperature for 24 to 48 h. After fixation, ganglia were dehydrated in an alcohol series ending with two 30-min washes of 100% ethanol. Ganglia were incubated in two 30-min washes of 100% xylene and transferred to Paraffin wax (Sigma-Aldrich) at 60°C.

A Leica Reichert-Jung 820-II Histocut Microtome was used to section metathoracic ganglia in wax blocks into 4-μm-thick slices. Sections were expanded by floating on distilled water at ~58°C on a slide warmer and mounted onto poly-L-lysine slides (Sigma-Aldrich). Mounted slides were air dried at room temperature for 30 min and oven-dried in a 45°C incubation chamber overnight.

Before immunohistochemistry, the mounted slides were deparaffined by soaking in xylene (Sigma-Aldrich) for 30 min in room temperature and rehydrated by washing sequentially in 100%, 95%, 80% and 50% ethanol for 10 min each at room temperature. The slides were rinsed in distilled water followed by Tris-buffered saline-Triton X-100 (TBST) buffer (20 mM Tris, 137 mM NaCl, 0.10% Triton X-100, pH 7.5) for 5 min each. To unmask the antigen, the Na^+-K^+-ATPase, the slides were washed at 95°C for 30 min in a Tris-EDTA-Triton X-100 buffer bath (10 mM Tris, 1 mM EDTA, 0.05% Triton X-100, pH 9.0), promoting the antigen-antibody binding ability. Finally, the slides were rinsed three times using TBST buffer for 5 min each and then background blocked in 5% normal goat serum (NGS) (Sigma-Aldrich), diluted using the TBST buffer, for 1 h at room temperature.

The Na^+-K^+-ATPase was tagged by incubating the slides with a primary mouse monoclonal antibody raised against the α_5-subunit of the Na^+-K^+-ATPase from chicken (purchased from Developmental Studies Hybridoma Bank at the University of Iowa; see e.g., Homareda and Otsu 2013) at 5% dilution (TBST buffer and 5% NGS as a diluent) for 48 h at 4°C. Excess primary antibodies were washed off with three 5-min washes of TBST buffer. Localization of the Na^+-K^+-ATPase was visualized by incubating in a Goat Anti-Mouse IgG (whole molecule)-fluorescein isothiocyanate (FITC) conjugated secondary antibody (Sigma-Aldrich) as the secondary antibody diluted to a 1:100 ratio (TBST buffer and 5% NGS as the diluent) for 24 h at 4°C.

To reveal DNA in nuclei, sections were double-labeled with 4′,6-diamidino-2-phenylindole (DAPI) for 5 min at room temperature with a concentration of 0.1 μg DAPI/ml in distilled water, followed by two 5-min rinses in TBST buffer. The slides were sealed using SlowFade Gold Antifade kit (Sigma-Aldrich) to minimize the fluorescence fading.

Sections were examined using an Axioplan 2 Imaging Microscope (Carl Zeiss MicroImaging), and images were taken with a Leica DC 500 digital camera (Spectronic Camspec), and saved using Openlab 4.0.1 (developed by Improvision PerkinElmer). Data from each ganglion were collected within the same session using the same gain and settings to avoid discrepancies in fluorescence intensity. Negative control sections, prepared by omitting the primary antibody, displayed only dim background fluorescence, confirming binding specificity of the FITC antibody to the primary antibody.

Images for nine control ganglia and nine HS preconditioned ganglia were analyzed using ZEN 2009 Light Edition (Carl Zeiss MicroImaging). Three neuronal somata that appeared representative of the level of fluorescence in the section were selected from each metathoracic ganglion by visual inspection to quantify the localization of the Na^+-K^+-ATPase. FITC fluorescence intensity of the nucleus, cytoplasm and plasma membrane was measured at three different cursor locations for each neuron and averaged. Since the nuclei were presumably absent of Na^+-K^+-ATPase, the membrane and cytoplasmic FITC intensities were normalized relative to nuclear intensities of each neuron to control for variation in background fluorescence.

Statistics. Data were tested for normality (Shapiro-Wilk test) and equal variance (Levene median test), and appropriate tests were applied using statistical software in SigmaPlot 11.0 (Systat Software). For comparison of two groups, Student's *t*-tests were used for parametric data and Mann-Whitney rank sum tests were used for nonparametric data. For comparison of more than two groups, two-way ANOVA were used, followed by Holm-Sidak multiple-comparison tests, and nonparametric data were logarithmically transformed to satisfy the assumptions of the tests. Correlations were tested using the Pearson product moment correlation.

Data were plotted using SigmaPlot. For parametric data, error bars represent the SE of the means. Nonparametric data are presented as box plots of 25th to 75th percentiles with a line showing medians, with whiskers to the 10th and 90th percentiles and outliers as individual points. Significance was assessed at $\alpha = 0.05$. Significantly different data ($P < 0.05$) are indicated by lettering (columns with different letters are different) or by asterisks when the comparison is between a treatment and a control.

RESULTS

Anoxic coma in whole animals. Locusts immersed in water struggled for a period of time, searching for a substrate with the

prothoracic and mesothoracic legs, using both hind legs to kick vigorously and generating abdominal ventilatory pumping contractions. Movements became less coordinated, and entry into coma was timed as occurring when the limbs quivered and extended, suggesting uncontrolled motoneuron discharge, followed by a lack of motion, including cessation of abdominal contractions. On return to air, the first signs of recovery were usually ventilatory movements of the abdomen, followed by pumping movements of the head relative to the thorax. The first abdominal movements could be small and difficult to detect; however, recovery of the ability to stand was abrupt as locusts righted themselves and adopted a stable stance within 1 or 2 s.

Male locusts entered coma earlier than females (male: 1.7 min; female: 3.7 min; reported here and below as medians of 10 males and 10 females in each of the control and HS conditions, see Fig. 1 for box plots) and took longer to recover ventilation (male: 15.4 min; female: 9.0 min) and to stand (male: 39.7 min; female: 29.0 min). In both males and females, prior HS increased the time to enter a coma (male: 3.0 min; female: 4.3 min), increased the time to recover ventilation (18.2 min; female: 13.2 min) and decreased the time to stand (male: 37.3 min; 25.0 min).

For statistical comparisons, two-way ANOVAs were performed on $\log_{10}$ transformed data. For time to succumb, there were strong effects of sex ($P < 0.001$) and HS treatment ($P < 0.001$) with a significant interaction between these variables ($P = 0.008$). Subsequent pairwise Holm-Sidak comparisons showed that sex had a significant effect on time to succumb in both control and HS conditions, but that HS had a significant effect in males but not in females. For time to ventilate, there were strong effects of sex ($P < 0.001$) and HS treatment ($P < 0.001$) with no interaction between these variables ($P = 0.137$). For time to stand, there was a strong effect of sex ($P < 0.001$) a significant effect of HS treatment ($P = 0.017$) and no interaction between these variables ($P = 0.739$).

To reduce variability in the datasets and to enable comparisons with published research primarily on male locusts, we focused our subsequent efforts on males only. Closer examination of the data showed that the control and HS groups could be clearly separated by comparing two variables together (Fig. 2). In both groups, there was a negative correlation between the time to succumb and the time to start ventilation (Fig. 2*A*; control: $r = -0.8$, $P = 0.005$; HS: $r = -0.7$, $P = 0.014$). However, only HS locusts showed a negative correlation between the time to succumb and the time to stand (Fig. 2*B*; control: $P = 0.5$; HS: $r = -0.8$, $P = 0.004$).

Anoxic coma in dissected preparations. Application of sodium azide to a dissected preparation caused a rapid cessation of ventilatory movements of the abdomen, coincident with a silencing of rhythmical motor activity recorded electromyographically (see also Rodgers et al. 2007). Ventilatory motor activity recovered when the preparation was reperfused with fresh saline. HS increased the rate of recovery from azide-induced anoxic coma (control: 15.3 ± 1.1 min, $n = 10$; HS: 12.1 ± 1.4 min, $n = 10$; $P < 0.05$, *t*-test; not shown).

We confirmed in a dissected preparation that the anoxic coma induced by immersion generated a similar surge of $[K^+]_o$ as has been described for the azide-induced coma in a dissected preparation (Rodgers et al. 2007) (Fig. 3*A*). To compare effects of drugs and pretreatments on the rate of $[K^+]_o$ clearance, we

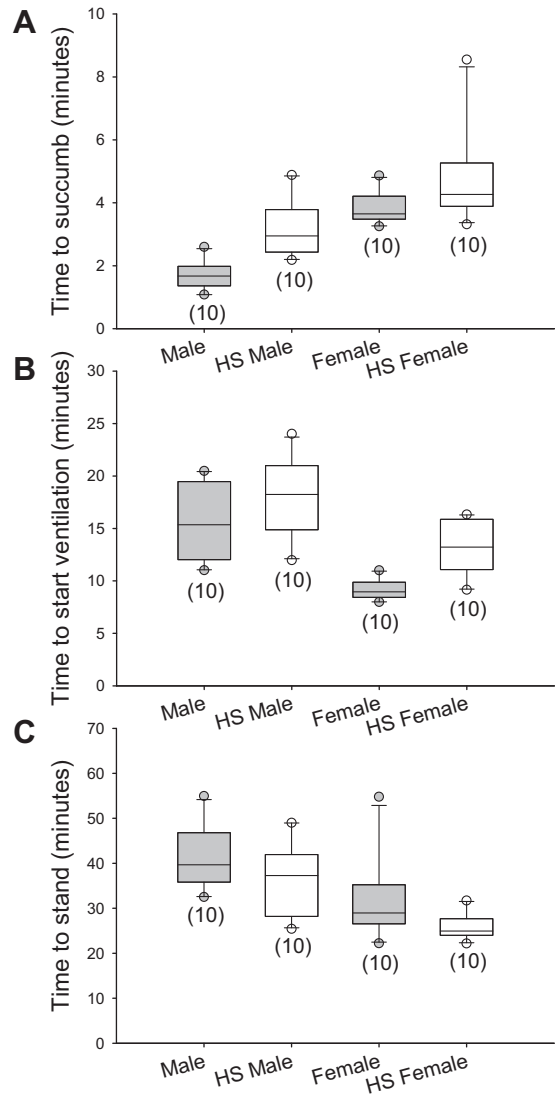

Fig. 1. Effects of prior heat shock pretreatment (HS) on anoxic coma induced by suffocation under water. *A*: HS increased the time to succumb after immersion under water. There was an overall significant effect of HS that was driven by the difference in males. *B*: HS increased the time to start ventilation after removal from the water. There are statistically significant effects of sex and HS and no significant interaction between them. *C*: HS decreased the time to stand after removal from the water. There are statistically significant effects of sex and HS and no significant interaction between them. Numbers in parentheses indicate sample sizes.

used controlled injections of 50 nl of 150 mM KCl into the neuropile of the metathoracic ganglion (Fig. 3*B*), as described previously (Armstrong et al. 2009), and measured the rate of recovery of the ventilatory motor pattern.

$[K^+]_o$ dynamics in dissected preparations. HS had no effect on the latency from KCl injection into the metathoracic ganglion to the surge in $[K^+]_o$ that occurs at the time of motor pattern arrest. However, HS shortened the duration of the $[K^+]_o$ surge measured at its half-amplitude (median of control: 1.9 min; HS: 1.4 min; $P = 0.009$, Mann Whitney rank sum test). These data indicate that the K^+ disturbance is cleared more rapidly after HS.

HS also shortened the time it took for ventilatory motor activity to resume as $[K^+]_o$ was cleared from the extracellular space (Fig. 4*A*; median of control: 2.6 min; HS: 2.0 min). Note

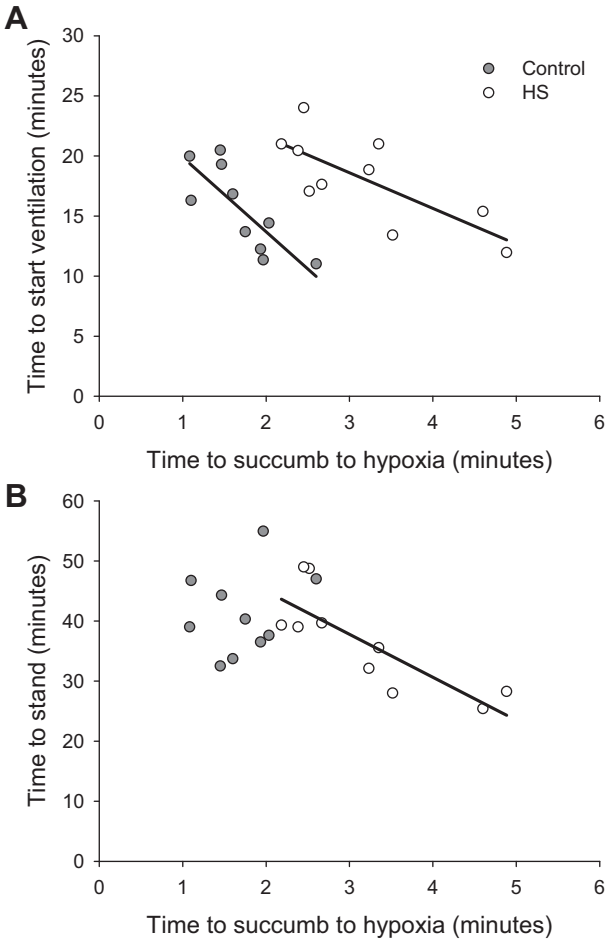

Fig. 2. Negative correlation between the time taken to succumb to anoxia and the time taken to recover in males. *A*: in control and HS male locusts, shorter times to succumb are associated with longer times to recover ventilation. *B*: in HS but not in control male locusts, shorter times to succumb are associated with longer times to stand after removal from the water.

that the time course of motor pattern recovery is considerably shorter than the time course of recovery of whole-animal ventilation. This can likely be accounted for by different durations of the disturbances: 30 min of whole-animal anoxia vs. about 2 min of ventilatory arrest during a KCl-induced $[K^+]_o$ surge. Moreover KCl-induced arrests would not be accompanied by any of the metabolic disturbances of a prolonged anoxic coma. Manipulation of PKG activity has effects on KCl-induced $[K^+]_o$ surges and on recovery from immersion anoxia in whole animals (Armstrong et al. 2009; Dawson-Scully et al. 2010), so we compared the effect of HS with the effect of PKG pharmacology. Inhibition of PKG with KT5823 had a similar effect to HS (Fig. 4*A*; median: 2.2 min), but potentiation of PKG by preventing its breakdown using the phosphodiesterase inhibitor, T-0156, was not different from control, although it was different from both HS and KT5823 conditions (median: 3.8 min) (Kruskal-Wallis one-way ANOVA on ranks, $P < 0.001$; followed by Dunn's all pairwise multiple comparisons).

PKG inhibition with KT5823 had the same effect as heat shock on time to recover. Inhibition of PKG activity is also associated with thermotolerance (Dawson-Scully et al. 2007). Given that a prominent effect of HS is also thermotolerance, we wondered whether the effects of HS could be mediated by a reduction in PKG activity. Thus we compared PKG activity in separate ganglia from 12 control and 12 heat-shocked animals and found that HS increased the specific activity of PKG in the meso- and metathoracic ganglia (Fig. 4*B*; control: 32.1, HS: 43.4 pmol·min^{-1}·mg protein^{-1}; $P = 0.03$, *t*-test). This suggests that the HS mechanism for increasing the rate of $[K^+]_o$ clearance and recovery of motor pattern generation is independent of any role that acute PKG inhibition might have.

Na^+-K^+-ATPase activity, abundance and distribution. Our laboratory has previously reported that HS had no effect on Na^+-K^+-ATPase activity in the metathoracic ganglion using small samples ($n = 6$ control and 6 HS; Rodgers et al. 2007). We reexamined this with larger sample sizes of 24 in each of the control and HS groups and found that HS did have a mild effect on pump activity, increasing it from a median of 62.2 nmol ATP·min^{-1}·mg protein^{-1} to 76.2 nmol ATP·min^{-1}·mg protein^{-1} (Fig. 5*A*; Mann-Whitney rank sum test, $P = 0.001$). This increase in activity could not be accounted for by an increase in the abundance of the protein. Densitometry analysis of Western blots (Fig. 5*B*) revealed that there was no difference in amount of Na^+-K^+-ATPase α-subunit label relative to α-tubulin label between three samples taken from a homogenate of six control ganglia and three samples taken from a homogenate of six HS ganglia ($P = 0.78$). The negative statistical result should be treated with caution because of the low sample size and low power of the test.

Localization of the α-subunit of Na^+-K^+-ATPase was determined immunohistochemically (Fig. 6). Preparations lacking the primary antibody had almost undetectable background fluorescence (not shown). Strong labeling was observed in the perineurial sheath of the ganglion and in axon profiles of cross-sectioned nerve roots. Clusters of neuronal somata (Fig. 6*A*, box; Fig. 6*Bii*) could be easily discerned in coronal

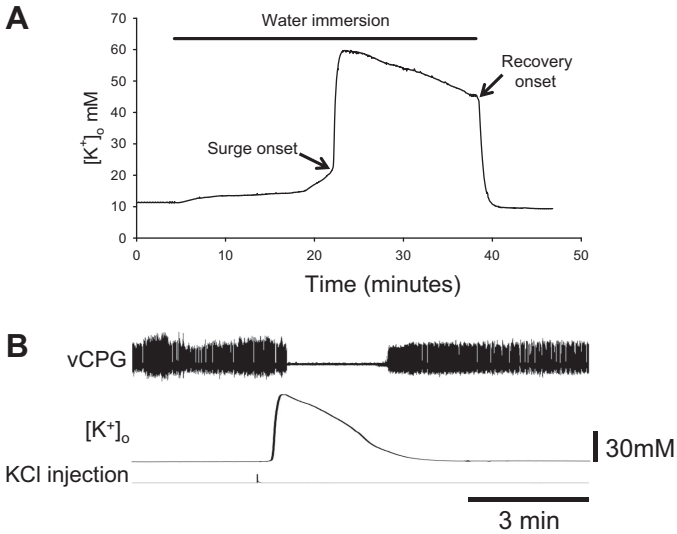

Fig. 3. Surges of extracellular potassium ion concentration ($[K^+]_o$) recorded in the metathoracic ganglion induced by water immersion and triggered by injection of high $[K^+]$ saline. *A*: sample trace of $[K^+]_o$ recorded from the metathoracic ganglion. The line (water immersion) indicates when the preparation was submerged under saline. An abrupt surge of $[K^+]_o$ (arrow at surge onset) returned to normal (arrow at recovery onset) when the saline was removed. *B*: similar surges of $[K^+]_o$ were induced by 50-nl injections of 150 mM KCl into the neuropile of the metathoracic ganglion. The $[K^+]_o$ surge was associated with arrest of ventilatory central pattern generator (CPG) activity.

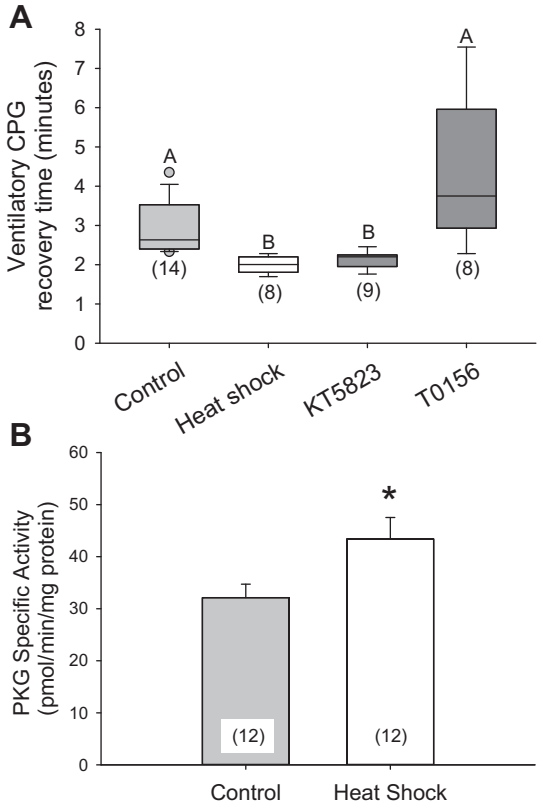

Fig. 4. Prior HS and inhibition of protein kinase G (PKG) shortened the time taken for the ventilatory CPG to recover. *A*: prior HS shortened the time taken for the ventilatory CPG to recover, and this was mimicked by treatment with 10 μM KT5823. There was no significant effect of 100 μM T-0156 on recovery time compared with controls, but there was compared with HS and KT5823 preparations. [A,B]|Significance indicated by letters above the box plots where plots with the same letter designation are not significantly different. *B*: HS significantly increased activity of PKG in meso- and metathoracic ganglia. *Significant difference of HS from control. Numbers in parentheses indicate sample sizes.

sections, and the nuclei were generally devoid of label (compare Fig. 6, *Bi* and *Bii*). Glial somata were difficult to resolve in these sections, and for convenience we examined the same cluster of neuronal somata of the first abdominal neuromere in different preparations. Somata in preparations from HS animals appeared to have more intense labeling in the plasma membrane of neurons than in preparations from control locusts (Fig. 7). For nine control and nine HS preparations, we quantified the intensity of label in cytoplasm and plasma membrane relative to the label in nuclei of the same neuronal somata (see MATERIALS AND METHODS; Fig. 8). The plasma membrane had more label relative to the nucleus than cytoplasm did (control cytoplasm median: 1.35; control membrane median: 2.22; $P < 0.001$, Mann-Whitney rank sum test). HS increased levels in cytoplasm (HS cytoplasm median: 1.57; $P = 0.042$ compared with controls, Mann-Whitney rank sum test). The most dramatic effect of HS was in the relative level of label in the neuronal plasma membrane (HS membrane median: 5.87; $P < 0.001$ compared with control membrane, Mann-Whitney rank sum test).

DISCUSSION

Entry into coma is an adaptive strategy for coping with periodic inundation by shutting down energetically expensive neural and muscular function (Petillon et al. 2009). The timing of an anoxic coma is primarily controlled by properties of the nervous system and is associated with the timing of triggered, all-or-nothing surges of $[K^+]_o$ in the neuropile (Armstrong et al. 2011; Rodgers et al. 2007). In locusts (Rodgers et al. 2010) and *Drosophila* (Armstrong et al. 2012), similar disturbances of neural K^+ homeostasis underlie chill coma, suggesting a common mechanism for shutting down neural activity. We have shown here that prior exposure to hyperthermia (HS) affected the timing of anoxic comas and the rate of clearance of $[K^+]_o$ from the neuropile. The same pretreatment increased the relative abundance of Na$^+$-K$^+$-ATPase subunits in neuronal plasma membranes. We conclude that HS caused trafficking of the Na$^+$-K$^+$-ATPase, and we suggest that this provides a mechanism for modulating the timing of anoxic coma.

Under anoxic conditions, the metabolic rate of locusts measured by microcalorimetry shows a brief surge coincident with struggling, followed by a rapid drop to about 5% of the normal resting metabolic rate as the locust enters a coma (Weyel and Wegener 1996). The coma occurs before any appreciable drop of ATP levels in nervous tissue (Rodgers et al. 2007), although, if anoxia is maintained, ATP levels in the central nervous system drop to very low levels (Hochachka et al. 1993; Weyel and Wegener 1996). On return to normoxia, the increase in metabolic rate is almost instantaneous and shows a prominent surge coincident with recovery (Money et al. 2014; Moratzky et al. 1993). However, restoration of ATP levels and clearance of metabolites associated with glycolysis takes time, and the

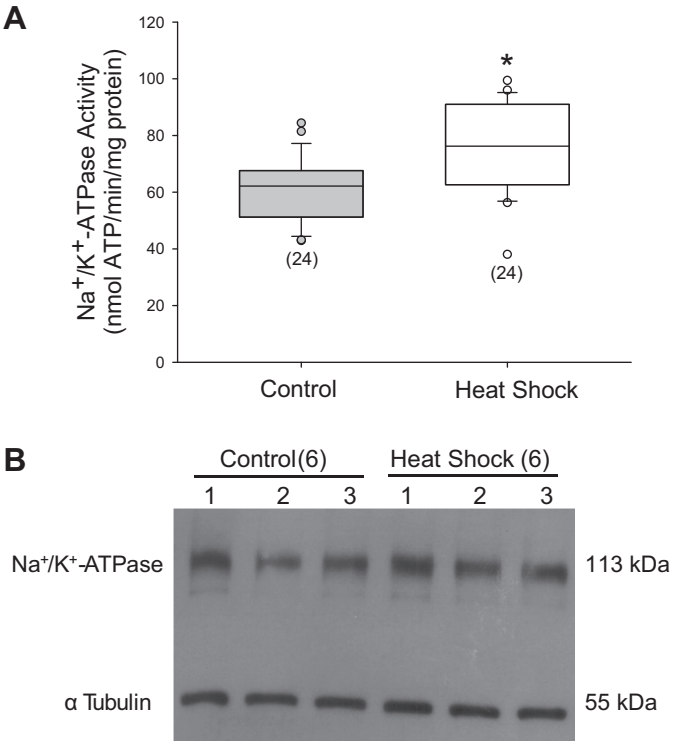

Fig. 5. Prior HS increased Na$^+$-K$^+$-ATPase activity in metathoracic ganglia but did not increase protein abundance. *A*: comparison of Na$^+$-K$^+$-ATPase activity from metathoracic ganglia of control and HS locusts. *Significant difference from control. *B*: three replicates of Western blots from homogenates of metathoracic ganglia from 6 control locusts and 6 HS locusts labeled with antibodies against the α-subunit of the Na$^+$-K$^+$-ATPase and α-tubulin. There was no significant difference in protein abundance. Numbers in parentheses indicate sample sizes.

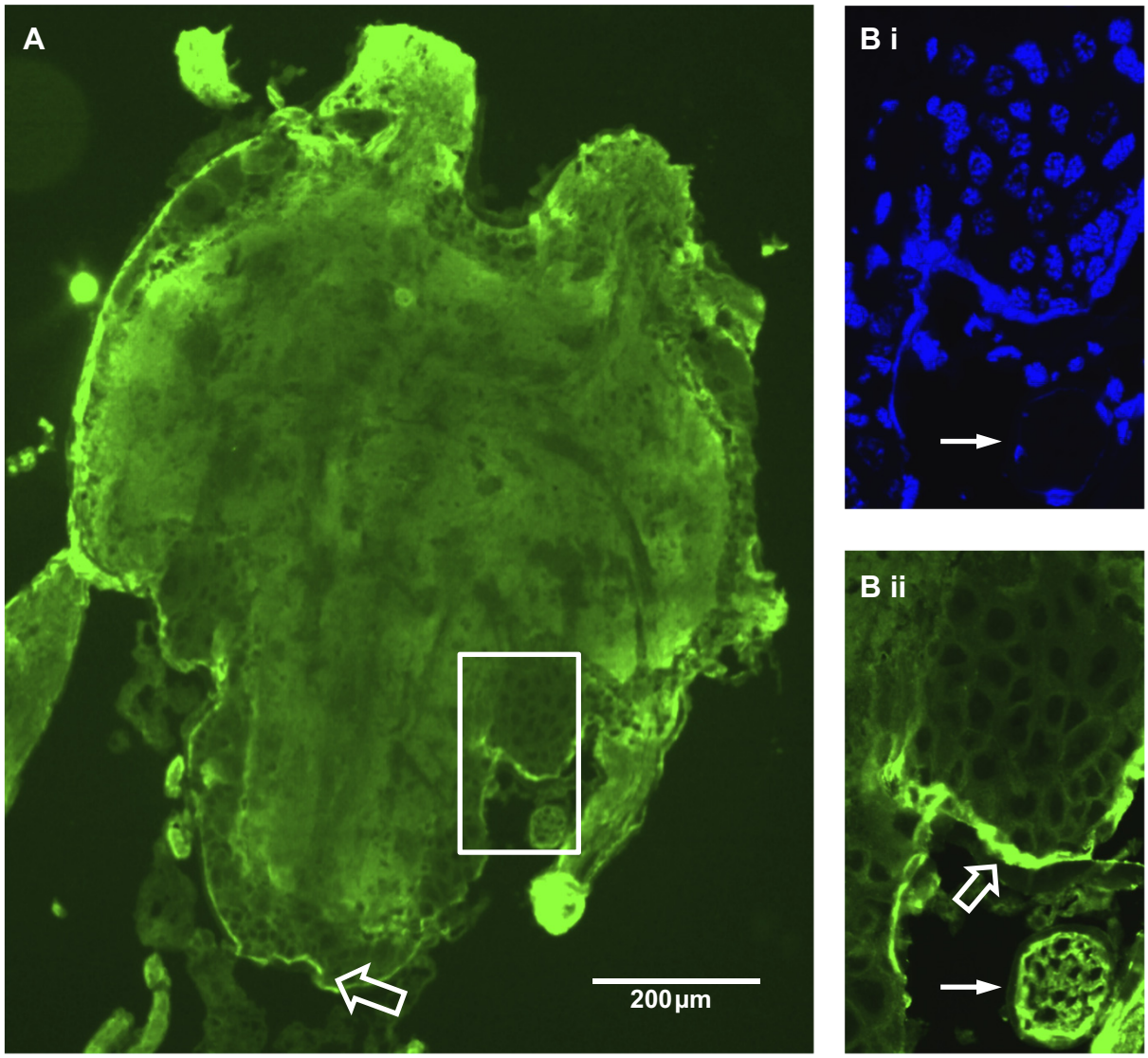

Fig. 6. Localization of the α-subunit of Na^+-K^+-ATPase in coronal sections of the metathoracic ganglion. *A*: whole metathoracic ganglion oriented with the anterior connectives toward the top and the third abdominal neuromere at the bottom. Open arrow indicates a high density of label in the perineurial sheath at the edge of the section. The white box has been enlarged in *Bi* and *Bii*. *Bi*: DAPI stain to locate cell nuclei. *Bii*: enlargement illustrates high density of label in perineurial sheath (open arrow) and in axonal membrane profiles of a nerve root (solid arrow). Note that the nerve root has no nuclei (see solid arrow in *Bi*), which show only background levels of labeling (compare *Bi* and *Bii*).

size of the metabolic disturbance is proportional to the duration of the anoxia (Hochachka et al. 1993; Weyel and Wegener 1996). Recovery of movement and climbing ability in *Drosophila* takes a time proportional to the duration of the anoxic coma, and this is associated with the time taken for neuromuscular function to recover (Krishnan et al. 1997). After 30 min of anoxia, a giant fiber-evoked muscle action potential can be recorded 14 min after a return to normoxia, whereas complete recovery of the action potential amplitude takes 40 min. For our experiments, entry into coma would likely have been determined primarily by the reserve of air in the tracheal system and air sacs and the rate at which oxygen was consumed. The rate of recovery would likely have been associated with the magnitude of the metabolic disturbance and the time taken to clear it from the central nervous system and the musculature. Tissues with a relatively larger mass-specific metabolic rate like nervous tissue will experience relatively larger metabolic disturbances. Simple movements that require little neural coordination and relatively little muscle strength (e.g., ventilation) are expected to recover before more complex activities that require sophisticated neuromuscular coordination and strength (e.g., righting to a standing position).

In our experiments, there was a clear difference in the absolute measures of sensitivity to suffocation in male and female locusts. Females from our colony have about 1.75 times the body mass of males (males: 1.6 g; females: 2.8 g) (Rodgers et al. 2006), and metabolic rates scale allometrically with body size (Darveau et al. 2002), suggesting that the sex differences might be accounted for by size differences. Another possibility is that female locusts were more sluggish and struggled less after immersion, although such a difference was not obvious during the experiments. These are possibilities that we did not explore. HS had similar effects in males and females, and so, to reduce variability in the data, we focused our attention on the effects of HS on males.

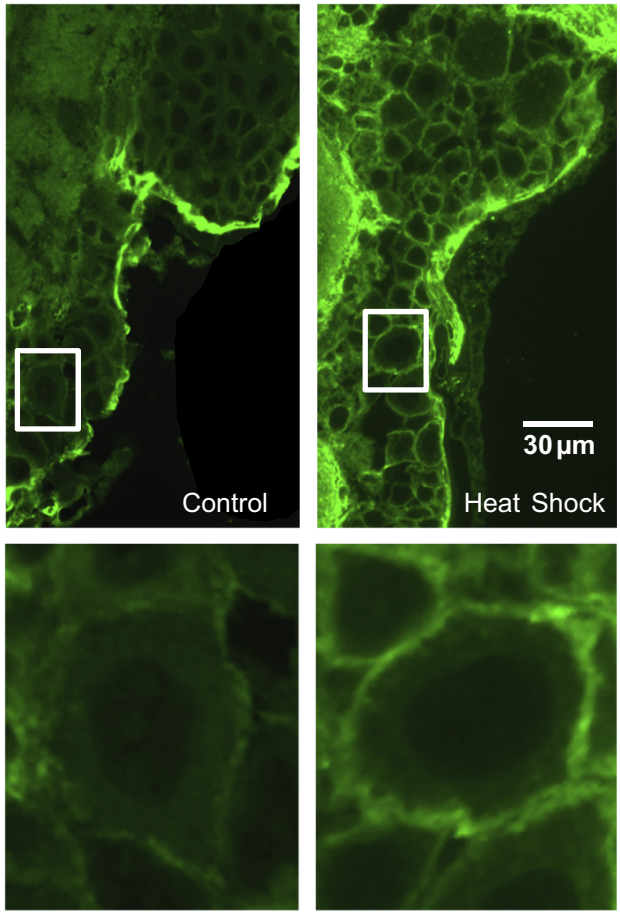

Fig. 7. Effect of HS on localization of Na^+-K^+-ATPase in neuronal somata. Panels on the *left* are from the metathoracic ganglion of the control preparation shown in Fig. 7. Note that, for clarity, the bright label associated with the nerve roots has been masked. Panels on the *right* are from a similar region of the ganglion in a HS preparation. The *lower* panels are enlargement of the white boxes indicated in the *upper* panels to illustrate the distribution of label associated with a single neuronal soma. Different densities of label are associated with the nuclei (darkest, background labeling), cytoplasm and plasma membrane (brightest). Note that the labeling is more intense in the plasma membrane in the HS preparation.

HS had opposite effects on our measures of whole animal recovery: increasing the time to start ventilation and decreasing the time to stand. This implies that these measures incorporate different processes. Our observation that HS increased the abundance of Na^+-K^+-ATPase in neuronal membranes suggests that the increased ability to clear rising levels of $[K^+]_o$ would increase the time taken to reach the threshold for neural shutdown (thus increasing the time to succumb). It would also increase the mass-specific metabolic rate of the central nervous system, thus creating a greater metabolic disturbance under anoxic conditions (thus increasing the time to start ventilation). There was a correlation between an increased time to succumb and a decreased time to start ventilation in both control and HS locusts. This is consistent with the notion that locusts with a low neural metabolic rate would take longer to exhaust the remaining air supply and also have less of a metabolic disturbance to clear. Although HS increased the time to start ventilation in whole animals, it decreased the time to recover ventilatory pattern generation after an arrest induced by KCl injection into the metathoracic ganglion in a semi-intact preparation. These findings are not contradictory, because the latter experiments focused only on $[K^+]_o$ dynamics with none of the metabolic disturbances caused by anoxia. With this more reductionist approach, it becomes easier to accept a causal link between the effects of HS on Na^+-K^+-ATPase distribution and its effects on $[K^+]_o$ clearance.

The HS-induced reduction of the time for whole animals to recover the ability to stand can be interpreted as an improvement of repair processes that would speed the strengthening of neuromuscular function. Locusts express heat shock proteins (HSPs), such as HSP70 (Qin et al. 2003), whose expression is notably upregulated in the gregarious phase (Badisco et al. 2011; Wang et al. 2007). HS treatment similar to the one we used has no detectable effects on HSP70 expression in thoracic muscles or metathoracic ganglia (Dehghani et al. 2011). Nevertheless, this leaves a role for HS-induced upregulation of the other HSPs that could protect proteins against stress, or post-translational modifications mediated by a heat-induced release of nitric oxide (Armstrong et al. 2009) and the increased PKG activity that we observed after HS. It is clear, however, that the more rapid recovery of potassium ion homeostasis induced by PKG inhibition (Armstrong et al. 2009) represents a mechanism different from the more rapid recovery of ion homeostasis after HS (Rodgers et al. 2007), which we found here to be associated with an increase in PKG activity. The time courses of the two mechanisms are also different, with PKG inhibition being fast-acting, whereas the effects of HS are seen 4 h from the start of the treatment. Thus the effect of HS cannot be attributed to a reduction in PKG activity.

Previously, our laboratory has shown that HS has a long-term effect of reducing whole cell K^+ currents recorded from

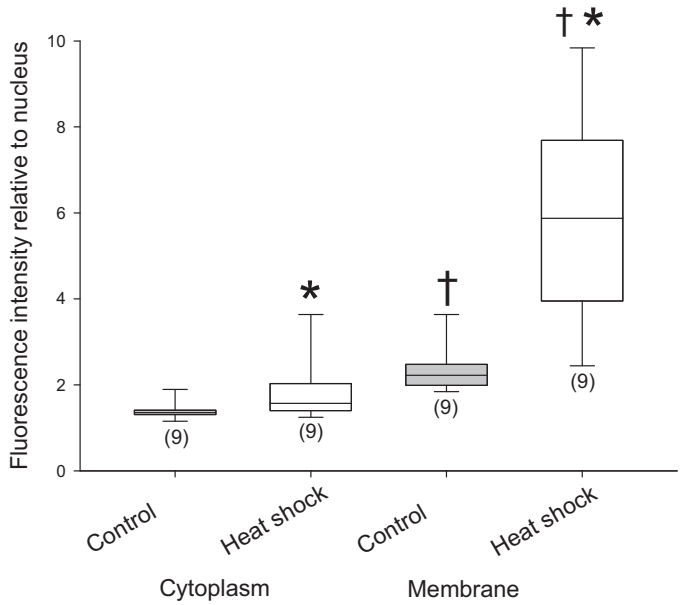

Fig. 8. HS induces trafficking of the Na^+-K^+-ATPase to plasma membranes of neuronal somata. In 9 control and 9 HS metathoracic ganglia, the intensity of fluorescence in cytoplasm and plasma membrane was quantified (see MATERIALS AND METHODS) and expressed relative to background label over the nuclei. Note that there was more label associated with the plasma membrane than with cytoplasm, and that HS increased levels marginally in the cytoplasm and considerably in the plasma membrane. †Significant difference between levels of label in cytoplasm and plasma membrane within control or HS. *Significant differences of HS compared with control values within cellular location. Numbers in parentheses indicate sample sizes.

neuronal somata in slices prepared from the metathoracic ganglion (Ramirez et al. 1999), and that this is associated with an increase in action potential duration that is protective at high temperatures (Wu et al. 2001). The present results show that the reduction of K^+ currents after HS is accompanied by trafficking of Na^+-K^+-ATPase to the plasma membrane of neuronal somata. Both of these effects would contribute to protecting ion homeostasis in the nervous system from stress and would reduce the tendency for neurons to become hyperexcitable with a build-up of $[K^+]_o$. Longer term metabolic consequences of prolonged anoxia are not protected by HS, and after 4 h of anoxia in a 100% N_2 atmosphere the recovery of the ability to stand was slower after HS (Wu et al. 2002). Such impairment was avoided in this study by restricting the duration of anoxia to 30 min. Moreover, N_2-mediated anoxia may have different metabolic consequences compared with anoxia by suffocation under water.

Trafficking of Na^+-K^+-ATPase in and out of cellular plasma membranes is well described as a means of coping with stress and modulating energy consumption (Alves et al. 2010; Benziane et al. 2009; Benziane and Chibalin 2008; Liu 2006). Under different circumstances in a freeze-tolerant insect, Na^+-K^+-ATPase is seasonally suppressed via phosphorylation mediated by kinases, including PKG (McMullen and Storey 2008). We found that HS induced a mild but significant increase in Na^+-K^+-ATPase activity in homogenates of metathoracic ganglia. Although the Western blots showed no effect of HS on the abundance of Na^+-K^+-ATPase, the immunocytochemistry showed that HS increased relative fluorescence in the neuronal cytoplasm as well as the plasma membrane, indicating increased abundance of the protein. We cannot rule out the possibility that posttranslational modifications contributed to the HS-induced Na^+-K^+-ATPase activity. However, we favor the interpretation that an increase in protein abundance, evident with immunocytochemistry, was not detected by Western blotting using a lower sample size. More importantly, HS induced a profound increase in the labeling of the Na^+-K^+-ATPase α-subunit in neuronal plasma membranes relative to labeling in the nuclei. For technical reasons, we could not measure relative labeling in glial cells, but, given the contribution of glial networks to K^+ homeostasis (Spong and Robertson 2013; Treherne and Schofield 1981), it would not be surprising to find similar trafficking induced by HS in glia. We suggest that the trafficking of Na^+-K^+-ATPase into plasma membranes after HS provides the nervous system with the means to combat ion disturbances during anoxia and to restore ion homeostasis more rapidly on return to normoxia.

In other studies, our laboratory has shown that anoxia or activation of the cellular energy sensor, AMP-activated protein kinase, has neural effects indicating a reduction of Na^+-K^+-ATPase activity (Money et al. 2014; Rodgers-Garlick et al. 2011). We predict that at least some of this downregulation is due to trafficking of the Na^+-K^+-ATPase out of neuronal membranes. These findings have been interpreted as a means of reducing energy consumption with a consequent loss of neural performance: an energy/performance trade-off. Thus there is increasing support for the notion that neural mechanisms are dynamically regulated by environmental conditions, enabling organisms to cope with the variable demands of their ecological niches.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: N.H., G.A.A., and M.C.-C. performed experiments; N.H., G.A.A., M.C.-C., and R.M.R. analyzed data; N.H., G.A.A., M.C.-C., M.B.S., and R.M.R. interpreted results of experiments; N.H., G.A.A., M.C.-C., M.B.S., and R.M.R. approved final version of manuscript; G.A.A. and R.M.R. conception and design of research; M.B.S. and R.M.R. edited and revised manuscript; R.M.R. prepared figures; R.M.R. drafted manuscript.

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