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Cold hardening modulates K^+ homeostasis in the brain of *Drosophila melanogaster* during chill coma

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ABSTRACT

Environmental temperature is one of the most important abiotic factors affecting insect behaviour; virtually all physiological processes, including those which regulate nervous system function, are affected. At both low and high temperature extremes insects enter a coma during which individuals do not display behaviour and are unresponsive to stimulation. We investigated neurophysiological correlates of chill and hyperthermic coma in *Drosophila melanogaster*. Coma resulting from anoxia causes a profound loss of K^+ homeostasis characterized by a surge in extracellular K^+ concentration ($[K^+]_o$) in the brain. We recorded $[K^+]_o$ in the brain during exposure to both low and high temperatures and observed a similar surge in $[K^+]_o$ which recovered to baseline concentrations following return to room temperature. We also found that rapid cold hardening (RCH) using a cold pretreatment (4 °C for 2 h; 2 h recovery at room temperature) increased the peak brain $[K^+]_o$ reached during a subsequent chill coma and increased the rates of accumulation and clearance of $[K^+]_o$. We conclude that RCH preserves K^+ homeostasis in the fly brain during exposure to cold by reducing the temperature sensitivity of the rates of homeostatic processes.

1. Introduction

As temperature falls insects enter a state of torpor called chill-coma from which they can recover if warmed (Rossbach, 1872; Mellanby, 1939; Kerkut and Taylor, 1958). The sensitivity of insects to cold can be reduced if animals are pre-exposed to cold temperature either chronically (cold-acclimated) or acutely (RCH) (Mellanby, 1939; Lee et al., 1987a; Chen and Walker, 1994; Rako and Hoffmann, 2006). Although much research has explored the molecular and metabolic changes induced following exposure to cold (Michaud and Denlinger, 2004; Qin et al., 2005; Norry et al., 2007; Overgaard et al., 2007; Sinclair et al., 2007; Telonis-Scott et al., 2009; Colinet et al., 2010a; Teets et al., 2012) the physiological changes underlying RCH remain unknown.

In addition to chill-coma, insects enter a reversible coma characterized by unresponsiveness to stimuli and cessation of behavior, in response to hyperthermia and hypoxia. In the migratory locust (*Locusta migratoria*), there is a profound and sudden loss of potassium ion homeostasis accompanied by neuronal depolarization in the central nervous system (CNS) during hyperthermic or anoxic stress. For example, at room temperature extracellular potassium ion concentration ($[K^+]_o$) in the metathoracic ganglion (MTG) is low (~10 mM); however, as thoracic temperature is ramped from 20 to 45 °C, there is a sudden and rapid rise of $[K^+]_o$ (occurring around 40 °C) to a stable elevated level around 50 mM which is maintained for the duration of the thermal stress (Robertson, 2004; Rodgers et al., 2007). A similar disturbance occurs during anoxia using 100% N_2 gas. The peak $[K^+]_o$ reached during anoxia is higher (~80 mM) but nonetheless recovers to baseline levels when the locusts are returned to normoxia (Rodgers et al., 2007). Electrophysiological monitoring of the ventilatory motor pattern (coordinated by a central pattern generator in the MTG) during ramped thermal stress tests has revealed that failure of motor pattern activity is preceded by excitation of the rhythm, followed by arrhythmias in pattern generation and culminating in cessation of all activity with a modest depolarization of neurons coordinating the pattern (Armstrong and Robertson, 2006; Armstrong et al., 2006). Failure of ventilatory pattern activity is tightly correlated with the rapid surge in $[K^+]_o$ and ventilatory pattern activity resumes when $[K^+]_o$ returns to baseline concentrations (Rodgers et al., 2007; Armstrong et al., 2009).

Chill-coma has traditionally been studied by monitoring the temperature at which insects are unable to stand and the time taken to recover the ability to stand on return to a permissive temperature (David et al., 1998; Udaka et al., 2010). More accurate measurements are possible by monitoring muscle potentials (Goller and Esch, 1990; Hosler et al., 2000) but such an approach may miss a potential role for the central nervous system in coordinating chill-coma. Preliminary results with locusts show

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that chilling results in CNS surges of $[K^+]_o$ similar to those evident during hyperthermia and anoxia suggesting similar underlying mechanisms (Rodgers et al., 2010). It seems clear that the mechanisms underlying chill-coma (MacMillan and Sinclair, 2011) have important functions in the CNS as well as in the periphery. The fact that the rate of recovery from chill-coma has a genetic basis (Udaka et al., 2010) supports the use of *Drosophila melanogaster* as a model organism for profitably investigating molecular mechanisms of RCH (Qin et al., 2005; Overgaard et al., 2007; Sinclair et al., 2007; Colinet et al., 2010a; Bing et al., 2012).

Surges of $[K^+]_o$ underlying nitrogen-induced comas can be measured precisely in the brain of *D. melanogaster* using K^+-sensitive microelectrodes (Armstrong et al., 2011). In this study we were interested in testing the hypothesis that RCH modifies cellular processes of ion regulation in the CNS during chill-coma. We monitored $[K^+]_o$ in the brain of *D. melanogaster* during exposure to environmental stressors to characterize K^+ disturbances that accompany the entry into and recovery from coma. Additionally, we examined RCH by determining the effect of prior cold shock (CS) on brain K^+ homeostasis during subsequent exposure to cold.

2. Materials and methods

2.1. Fly rearing

Flies of the Canton-S strain were raised on standard medium (0.01% molasses, 8.2% cornmeal, 3.4% killed yeast, 0.94% agar, 0.18% benzoic acid, 0.66% propionic acid) at 25 °C, 60–70% humidity and a 12 h/12 h (light/dark) light cycle with lights on at 0800 h. Flies were maintained at equal densities in plastic vials containing 5 ml of medium (approximately 20 adult flies/vial).

2.2. Electrophysiology

K^+-sensitive microelectrodes were fashioned from non-filamented glass pipettes (1 mm diameter; World Precision Instruments Inc., Sarasota, FL, USA), cleaned with methanol (99.9%), dried on a hot plate and pulled to form a low resistance (6–8 MΩ) tip. The microelectrodes were silanized by exposure to dichlorodimethylsilane (99%) (Sigma–Aldrich) vapour while baking on a hot plate (100 °C) for 1 h. After cooling, the microelectrodes were first back-filled to the tip with Potassium Ionophore I-Cocktail B (5% Valinomycin; Sigma–Aldrich) to form an artificial membrane permeable to K^+ and then back-filled with 500 mM KCl. The tips of the K^+-sensitive microelectrodes were suspended in distilled water until experimentation. Reference electrodes were made with filamented pipettes (1 mm diameter; World Precision Instruments Inc., Sarasota, FL, USA) pulled to form a low resistance (6–8 MΩ) tip and back-filled with 3 M KCl.

The K^+-sensitive and reference microelectrodes were inserted into electrode holders with chloride-coated silver wire, connected to a DUO 773 two-channel intracellular/extracellular amplifier (World Precision Instruments) and calibrated at room temperature (~20 °C). Two KCl solutions (15 and 150 mM) were used to determine the slope relationship of the electrode needed to calculate $[K^+]_o$ (mM) from the recorded voltage change using the Nernst equation (see Rodgers et al., 2007). Electrode sensitivities ranged from 50 to 60 mV for the 10-fold difference in K^+ concentrations and electrodes with sensitivities outside of this range were discarded.

All experiments were performed on male flies aged 3–7 days post-ecdysis. Flies were immobilized in a refrigerator (4 °C) for 3 min and then secured on a 0.5 cm diameter bed of wax on either a cover slip or on top of a recessed porous platform (6 × 12 cm, porous polyethylene) capable of delivering a laminar flow of N_2 gas over the entire fly (Armstrong et al., 2011). The brain was exposed by making a small (~200 μm sagittal slit in the cuticle along the dorsal midline of the fly head. K^+-sensitive microelectrodes were inserted through the sheath into the brain (protocerebral lobe) to measure $[K^+]_o$. A small drop of hemolymph-like solution, HL3 (Stewart et al., 1994), was carefully placed over the incision in the cuticle by sliding it down one of the glass electrodes.

2.3. Abiotic parameter manipulations

For cold shock pre-treatments, flies with food medium were placed in a refrigerator at 4 °C for 2 h and were allowed to recover for 1–2 h.

Rapid cooling and warming during recording was achieved by mounting the wax bed with the fly onto a peltier plate. Wax substrate temperature was monitored using a BAT-12 (Physitemp Instruments, Clifton, NJ, USA) with a thermocouple (24 gauge polyurethane coated wire with polyester insulated thermocouple bead) partially embedded in the wax next to the fly. Abdominal body temperatures were estimated in a subsequent set of experiments by correlating the temperature readout of a thermocouple placed in the wax substrate to an additional thermocouple placed within the abdomen of a fly while changing temperature. The relationship generated was used to estimate fly abdominal temperatures without the need to invasively position a thermocouple within the fly abdomen during experiments. Temperature was ramped up or down at a rate of 2.5 °C/min starting at room temperature (20–23 °C) until the surge of $[K^+]_o$ occurred. For cold stress experiments the temperature of the pinning bed was lowered from room temperature to 5 °C for 2–3 min before returning it to room levels. For heat stress experiments the temperature was ramped up until the surge in $[K^+]_o$ occurred and immediately returned to room levels. For both heating and chill experiments restoration to room temperature was achieved passively by switching off the peltier plate and allowing the wax bed to reach thermal equilibrium with room temperature. To generate an anoxia-induced surge in $[K^+]_o$ by suffocation the fly was submerged in saline to cover the spiracles. Three minutes following the surge in $[K^+]_o$ the saline pool covering the fly was removed. Anoxia-induced surges were also generated by passing a stream of N_2 gas over the preparations. Chemical anoxia was achieved by pressure-injection of small volumes (30 nl) of sodium azide (10 μM in HL3) directly onto the fly brain using a PicoSpritzer III (Intracel Inc., Shepreth, UK).

2.4. Statistical analysis

SigmaPlot 11.0 integrated with SigmaStat 3.1 was used to assess data groupings for significance. Statistical analyses used paired *t*-tests and one-way and two-way repeated measures ANOVA, followed by post hoc Tukey or Holm–Sidak multiple comparison tests. For non-parametric tests a Kruskal–Wallis one way ANOVA on ranks was performed. Significance was assessed at $p < 0.05$ (single asterisks or daggers) however the majority of *p*-values are less than 0.01. Error bars represent standard error measurement.

3. Results

In order to provide a better context for K^+ disturbances resulting from cold we examined the effects of several stressors known to cause loss of $[K^+]_o$ homeostasis in insects. We observed significant disturbances in $[K^+]_o$ homeostasis in the fly brain when it was exposed to hypothermia, hyperthermia or suffocation by saline immersion. Either cooling or heating the substrate from room temperature at a rate of 2.5 °C/min resulted in an abrupt surge in $[K^+]_o$ that returned to the pre-stressed concentration upon return to

room temperature (Fig. 1A and D). Twitching of the appendages was often observed near the moment of K^+ homeostasis loss. The accelerating increase in $[K^+]_o$ occurred at 8.3 ± 1.3 °C (n = 8) and at 41.0 ± 0.5 °C (n = 12) for chilling and heating experiments respectively. The peak $[K^+]_o$ reached during the surge was significantly higher than both the initial baseline and the $[K^+]_o$ after recovery for both treatments (paired t-test, $p < 0.05$) (Fig. 1D). The steady-state $[K^+]_o$ (measured immediately prior to returning to room temperature) was also significantly higher than baseline values before and after the applied stress and lower than the peak $[K^+]_o$ reached (paired t-test, $p < 0.05$). Cooling the substrate below 0 °C resulted in an elevated $[K^+]_o$ which did not return to pre-stressed levels on return to room temperature (n = 4, Fig. 1B). Similarly, in flies exposed to lethal heat (52.5 °C) for 2 min $[K^+]_o$ did not recover to baseline levels but remained elevated (40.0 ± 7.9 mM, n = 6) even after 10 min at room temperature. For comparison we also examined $[K^+]_o$ disturbance in the fly brain during exposure to hypoxia. Water immersion, exposure to N_2 gas or application of the mitochondrial electron transport chain inhibitor, sodium azide, all resulted in substantial disturbances in K^+ homeostasis with peak and steady state $[K^+]_o$ significantly higher than baseline and recovered $[K^+]_o$ (paired t-test, $p < 0.05$; Fig. 1C and D). We found that peak and steady state $[K^+]_o$ reached in flies exposed to N_2 gas were significantly higher than peak and steady state $[K^+]_o$ reached in flies exposed to cold and heat stress. As with exposures to cold, twitching of the appendages was observed in most of the animals examined at the point of entry into coma. These data indicate that during exposure to cold the fly brain displays a substantial disturbance in K^+ homeostasis, which is similar to the response to hyperthermia and hypoxia.

RCH increases survival following subsequent exposures to cold temperature (Lee et al., 1987b; Kelty and Lee, 2001) and shortens the time required for flies to stand following chill coma (Anderson et al., 2005; Mori and Kimura, 2008). To investigate if changes in brain K^+ homeostasis are altered following RCH induced by a CS pretreatment we recorded $[K^+]_o$ in the brain of CS-treated flies and exposed them to cold stress. Lowering the plate temperature at a rate of 2.5 °C/min resulted in $[K^+]_o$ surges in both CS-treated and Control flies (Fig. 2A). Although there was a general trend for CS-treated flies to display the rapid surge in $[K^+]_o$ at a lower temperature (6.4 ± 1.2 °C) during chilling there was no significant difference between CS-treated and Control flies (t-test, p = 0.5). Both peak and steady state $[K^+]_o$ were significantly higher than baseline and recovered $[K^+]_o$ in both Control and CS-treated flies (t-test, $p < 0.05$, Fig. 2B). However, CS-treated flies had a significantly higher peak $[K^+]_o$ than Control flies (Fig. 2B). The rates of $[K^+]_o$ accumulation and clearance were also significantly different in flies preconditioned with CS. At the start of chill coma CS-treated flies had a significantly faster rate of $[K^+]_o$ accumulation than Controls (one-way ANOVA, p = 0.04), and this was not significantly different from $[K^+]_o$ accumulation rates in flies exposed to N_2 gas (one-way ANOVA, p = 0.22; Fig. 3A). Also, at the end of the chill coma CS-treated flies displayed a faster clearance rate of $[K^+]_o$ (one-way ANOVA, p = 0.05; Fig. 3B) but both Control and CS-treated flies cleared $[K^+]_o$ slower than flies recovering from exposure to N_2 gas (one-way ANOVA, $p < 0.01$). The high rates of accumulation and clearance during N_2 anoxia is likely due to the fact that they were measured at a much higher temperature (~20 °C compared with 8 °C or 6 °C) and it has been established that chill coma recovery time is strongly temperature-dependent (David et al., 1998). To confirm that K^+ dynamics in the brain are temperature-dependent, in a separate experiment we measured N_2-induced $[K^+]_o$ surges at two different temperatures (23 and 16 °C). We found no significant difference in the rate of $[K^+]_o$ accumulation for this temperature difference (one-way ANOVA; p = 0.5; Fig. 3C). However, reduced temperature significantly reduced the rate of $[K^+]_o$ clearance at the end of an anoxic coma (one-way ANOVA; p = 0.015; Fig. 3D). These data demonstrate that flies pretreated with CS display an

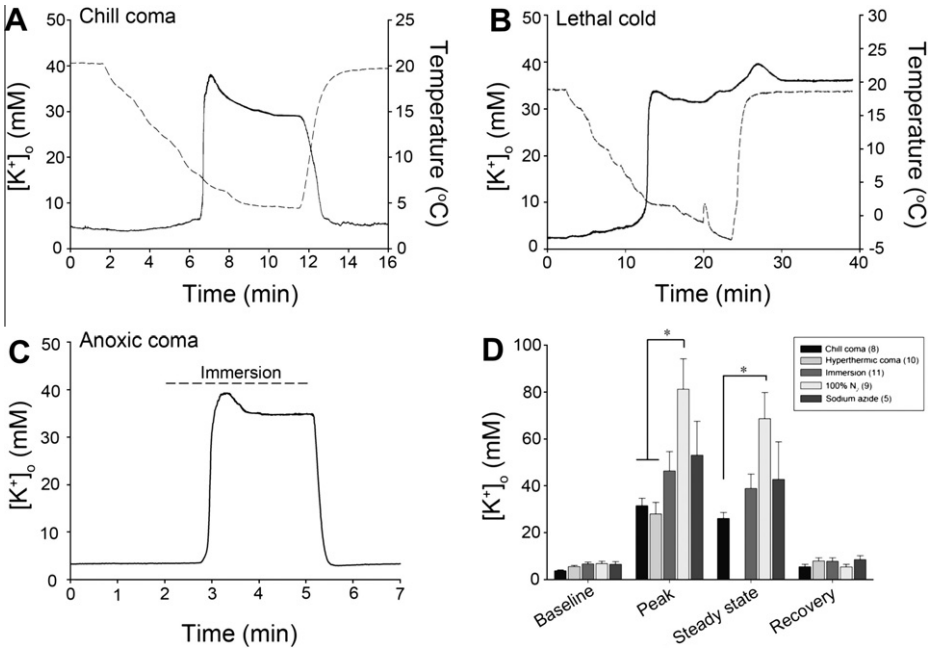

Fig. 1. Loss of K^+ homeostasis during coma. Abrupt surges in $[K^+]_o$ occur during stress and were coincident with coma and characterized by the cessation of movement and responsiveness to stimuli. (A) Example trace (sold line) of brain $[K^+]_o$ during chill coma. Dashed line indicates temperature. (B) Example trace of a fly exposed to lethal cold. Note the latent heat of crystallization occurring around the 20 min mark in the in the temperature trace. This fly did not recover K^+ homeostasis. (C) Coma in response to anoxia induced by immersion in saline (dashed line). (D) Summary data of $[K^+]_o$ surges including (in addition to the treatments in A and C): hyperthermia, anoxia via exposure to 100% nitrogen gas (N_2) and chemical anoxia via treatment with 10 μM sodium azide to block mitochondrial function. Within all treatments groups, peak and steady state $[K^+]_o$ were significantly higher than baseline and recovered $[K^+]_o$. Numbers in parentheses represent sample sizes and asterisks represent significant differences from the N_2-treated flies ($p < 0.05$).

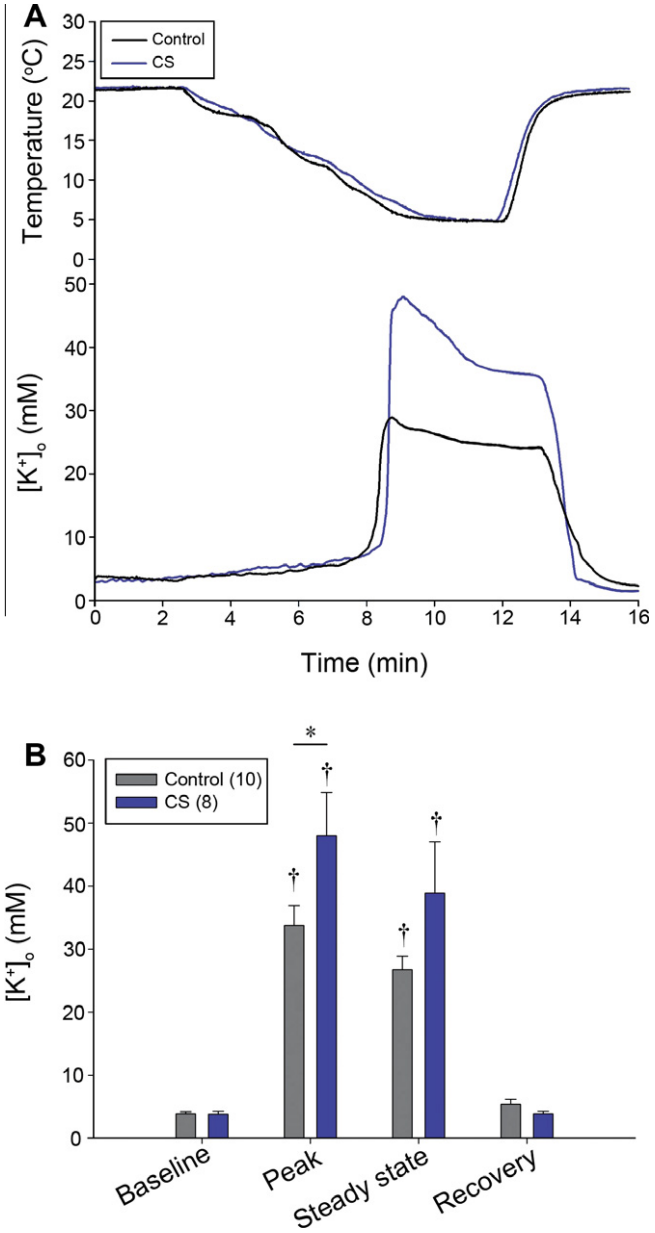

Fig. 2. CS pre-treatment alters $[K^+]_o$ accumulation during chill coma. (A) Example traces of brain $[K^+]_o$ of a CS-treated fruit fly and a control fly in response to a decreasing temperature ramp. All CS-treated flies displayed the rapid surge of $[K^+]_o$ and recovered to baseline levels when exposed to transient cold stress. (B) Tabulated data of $[K^+]_o$ of baseline, peak, steady state, and recovery in Control and CS-treated flies. Note that flies treated with CS displayed a larger peak $[K^+]_o$ compared to Control flies during cold stress. Numbers in parentheses represent sample sizes and asterisk represent significant differences from the Control flies, Daggers represent significant differences from baseline, steady state and recovery $[K^+]_o$ within Control and CS-treated flies ($p < 0.05$).

exaggerated loss of K^+ homeostasis with a faster onset and recovery of K^+ homeostasis during subsequent exposures to cold stress.

4. Discussion

In this study we used potassium ion-sensitive electrodes to measure changes in brain $[K^+]_o$ of restrained, minimally-dissected, *D. melanogaster* during exposure to various abiotic stressors. Following suffocation by saline immersion or exposure to 100% N_2 gas, sodium azide, heat stress or cold stress, a rapid rise in brain $[K^+]_o$ was observed in 100% of the animals. Both the peak and the steady state $[K^+]_o$ reached during exposure to 100% N_2 gas was significantly higher than those reached during exposure to all other forms of stress (Fig. 1D). A similarly large amplitude surge of $[K^+]_o$ following exposure to 100% N_2 gas has been observed in the MTG of *L. migratoria* (Rodgers et al., 2007) and in the brain of *D. melanogaster* (Armstrong et al., 2011). K^+ homeostasis was restored when the applied stress was removed and flies which maintained stable elevated $[K^+]_o$ in the brain after removal of the stress were considered moribund or dead. This was observed only after exposure to temperatures in excess of 50 °C and following freezing.

Disturbances of $[K^+]_o$ have been recorded from the locust MTG during cold exposure (Rodgers et al., 2010). Moreover surges in $[K^+]_o$ in the fly brain are coincident with an extracellular DC potential shift indicating a widespread depolarization of neurons (Armstrong et al., 2011). At the onset of coma tremors in the thorax, abdomen, legs, wings and antennae are routinely observed. These are likely caused by a short series of high frequency action potentials that occur in response to rises in $[K^+]_o$ which depolarize neuronal membrane potentials and open voltage-gated sodium channels. In the mammalian literature concerned with spreading depression, a similar phenomenon, they are referred to as prodromal spikes (Herreras et al., 1994; Müller and Somjen, 2000; Somjen, 2001). Spikes are routinely observed as short bouts (<10 s) of unpatterned high frequency activity during the onset of chill-coma (Hosler et al., 2000), hyperthermic coma (Rodgers et al., 2007) and when coma is induced by triggering a surge of $[K^+]_o$ via KCl injection into the neuropil in the MTG of the migratory locust (Armstrong and Robertson, 2006; Armstrong et al., 2006, 2009; Rodgers et al., 2010). Whereas some of this activity during chill-coma can be attributed to the depolarization of muscle membrane potentials and generation of muscle action potentials (Hosler et al., 2000) it is clear that neural failure in the CNS will also generate bursts of motoneuron action potentials (Wu et al., 2001; Rodgers et al., 2007).

Temperature has a direct effect on the ion channels and pumps involved in ion homeostasis in the CNS and neural failure at extreme temperatures can be attributed to mismatched temperature sensitivities of critical neural mechanisms (Robertson and Money, 2012). The result is an imbalance in processes of ion accumulation and clearance that leads to neuronal hyperexcitability and increasing $[K^+]_o$ in a positive feedback cycle that will terminate when all voltage-dependent Na^+ channels are open and inactivated (Armstrong et al., 2009). At this point K^+ ions will continue to flow through open voltage-dependent K^+ channels until the membrane potential matches the K^+ equilibrium potential. If the latter is more depolarized than the former then the K^+ current will be inward until equilibrium is reached and this would account for the drop in $[K^+]_o$ from a peak level to a steady-state level. The magnitudes of these $[K^+]_o$ surges, i.e. the magnitude of the ionic disturbance, will depend on the magnitude of the K^+ currents, which are in turn dependent on K^+ conductance and driving force, during the limited period from the threshold of the event to the subsequent inactivation of voltage-dependent Na^+ channels. Hence the larger amplitude surge associated with a N_2-induced coma indicates maximal K^+ flux during this period. If the event is triggered at high or low temperatures K^+ flux is reduced, likely due to both reduced conductance and reduced driving force, and the ionic disturbance will be reduced.

Loss of K^+ homeostasis was not prevented by pretreatment with CS as these flies displayed surges in $[K^+]_o$ during exposure to hypothermia, though there was a tendency for these to occur at lower temperatures. However, peak $[K^+]_o$ reached during chill-coma was significantly higher in CS-treated flies and rates of $[K^+]_o$ accumulation and clearance were both higher. As with other enzymatic reactions, reduced temperatures slow conformational changes in ion channel and pumps thereby impeding function (Hochachka

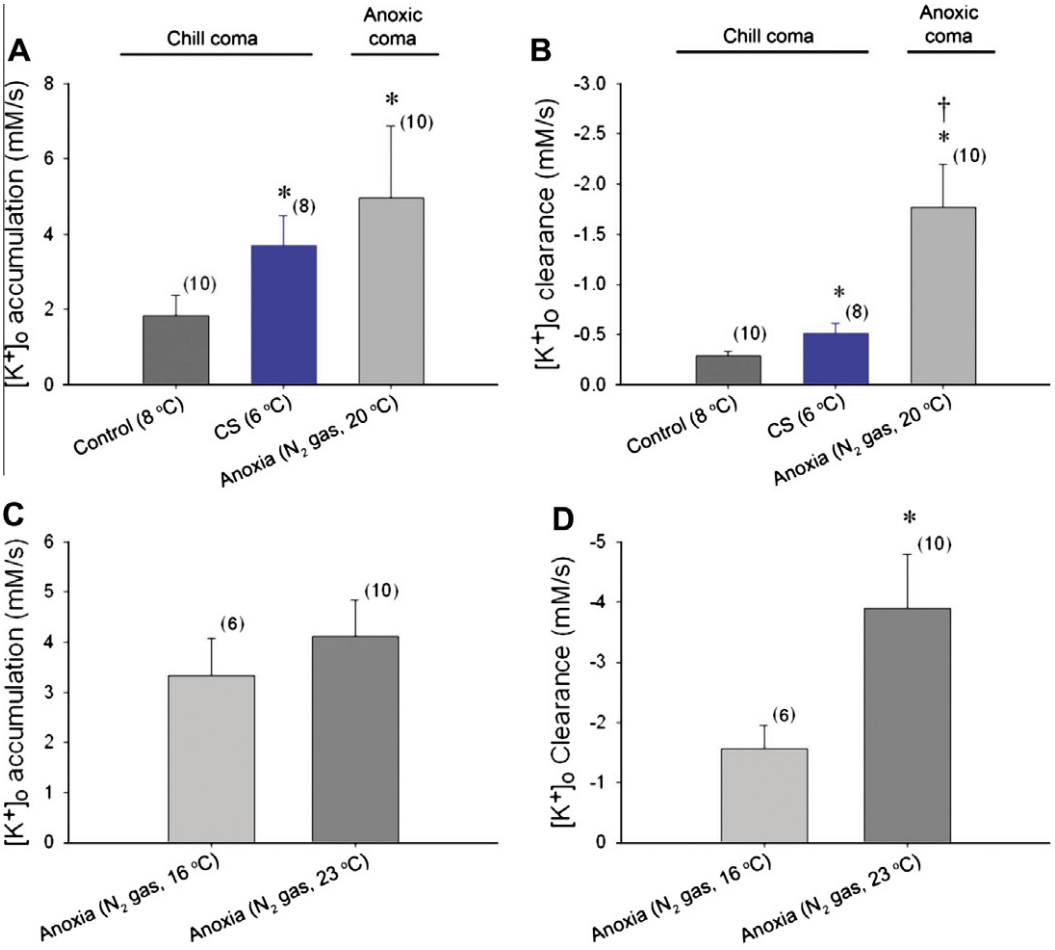

Fig. 3. CS preconditioning increases $[K^+]_o$ accumulation and clearance in the fly brain during exposure and recovery from cold stress respectively. (A) CS-treated flies accumulate $[K^+]_o$ significantly faster than Control flies during exposure to hypothermic stress. Accumulation of $[K^+]_o$ in the brain of CS-treated flies exposed to cold was not significantly different from accumulation of $[K^+]_o$ in flies exposed to anoxia. (B) Following removal of the cold stress CS-treated flies displayed significantly faster $[K^+]_o$ clearance rates than Control flies. However, both Control and CS-treated flies displayed slower clearance rates than flies recovering from anoxia. (C) The rate of $[K^+]_o$ accumulation at the onset of a N_2-induced coma was not affected by a 7 °C reduction in temperature. (D) The rate of $[K^+]_o$ clearance at the end of a N_2-induced coma was significantly reduced by a 7 °C reduction in temperature. Numbers in parentheses represent sample sizes and asterisks represent significant differences from the Control flies and daggers represent significant differences from CS-treated flies ($p < 0.05$).

and Somero, 1984; Rosenthal and Bezanilla, 2002; Galarza-Muñoz et al., 2011). Our results indicate that the CS-treatment generating the RCH response increased the efficacy of ion channels and the Na^+/K^+ ATPase allowing for improved function at lower temperatures. Thus the $[K^+]_o$ surge induced around 6 °C in CS-treated flies was closer in amplitude to the N_2-induced surge at room temperature in Control flies indicating that K^+ currents were temperature-compensated. Similarly the rate of K^+ clearance was faster after CS indicating that operation of the Na^+/K^+ ATPase was temperature-compensated. It is important to note that the rate of clearance after chill coma would have been affected by the low temperature at which it was recorded and the effect of CS was to make it closer to the clearance rate after N_2-coma at a higher temperature. Details about the mechanisms underlying this temperature compensation (phosphorylation, trafficking and membrane alterations) remain to be determined. Nevertheless it is noteworthy that physiological adaptations that enable neuronal operation in squids from different environmental temperatures involve modifications of ion conductances (Rosenthal and Bezanilla, 2002) and the Na^+/K^+ ATPase (Galarza-Muñoz et al., 2011) making them less sensitive to changes in temperature.

Much recent research has revealed the molecular changes that occur during RCH (Michaud and Denlinger, 2004; Qin et al., 2005; Norry et al., 2007; Sinclair et al., 2007; Yi et al., 2007; Telonis-Scott et al., 2009; Colinet et al., 2010b,c). Notably, there is increased expression of *Frost* (*Fst*) following exposures to cold (Goto, 2001; Qin et al., 2005; Udaka et al., 2010). Traditionally, the RCH response has been examined in animals held at sublethal hypothermia, whereas in our study we allowed a recovery period at a higher temperature between the cold pretreatment and our $[K^+]_o$ recordings in the brain. In similar experiments there is increased expression of Hsp23, Hsp26 and Hsp83 (Qin et al., 2005) and Hsp70 (Sinclair et al., 2007). In flies exposed to repeated bouts of anoxia, heat shock or tissue-specific expression of Hsp70 in glia stabilized resting baseline $[K^+]_o$ between exposures to anoxia (Armstrong et al., 2011). It is possible that Hsps might contribute to stabilizing ion channels and pumps thereby promoting ion homeostasis during and following hypothermic stress. However the exact mechanisms by which Hsps confer hypothermic tolerance are unknown. Robust expression of *Fst* occurs following exposure to cold (Colinet et al., 2010a) and its knockdown impairs recovery from chill coma. The role of *Fst* in maintaining ion homoeostasis has not been investigated and remains an interesting avenue for future investigations into the CS-induced changes in ion homeostasis underlying RCH. Whatever the molecular basis, we show here that an important mechanism of RCH in *D. melanogaster*

involves modification of the temperature sensitivities of processes of K^+ homeostasis in the CNS.

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