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## RESEARCH ARTICLE

# Protective effect of hypothermia on brain potassium homeostasis during repetitive anoxia in *Drosophila melanogaster*

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**SUMMARY**

**Oxygen deprivation in nervous tissue depolarizes cell membranes, increasing extracellular potassium concentration ($[K^+]_o$). Thus, $[K^+]_o$ can be used to assess neural failure. The effect of temperature (17, 23 or 29°C) on the maintenance of brain $[K^+]_o$ homeostasis in male *Drosophila melanogaster* (w1118) individuals was assessed during repeated anoxic comas induced by $N_2$ gas. Brain $[K^+]_o$ was continuously monitored using $K^+$-sensitive microelectrodes while body temperature was changed using a thermoelectric cooler (TEC). Repetitive anoxia resulted in a loss of the ability to maintain $[K^+]_o$ baseline at 6.6±0.3 mmol $l^{-1}$. The total $[K^+]_o$ baseline variation ($\Delta[K^+]_o$) was stabilized at 17°C (–1.1±1.3 mmol $l^{-1}$), mildly rose at 23°C (17.3±1.4 mmol $l^{-1}$), and considerably increased at 29°C (332.7±83.0 mmol $l^{-1}$). We conclude that (1) reperfusion patterns consisting of long anoxia, short normoxia and high cycle frequency increase disruption of brain $[K^+]_o$ baseline maintenance, and (2) hypothermia has a protective effect on brain $K^+$ homeostasis during repetitive anoxia. Male flies are suggested as a useful model for examining deleterious consequences of $O_2$ reperfusion with possible application for therapeutic treatment of stroke or heart attack.**

Key words: fruit fly, hyperthermia, insect, oxidative stress, reperfusion, temperature.

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## INTRODUCTION

Oxygen availability can be limited at the environmental or cellular level. Environmentally, animals can experience periods of hypoxia/anoxia because of flood-prone burrows or decreased partial $O_2$ pressure caused by increased altitude (Hoback and Stanley, 2001). At the cell level, the availability of $O_2$ can be reduced by heavy exercise or by disruptive physiological events like heart attack or stroke (Hochachka, 1998). Furthermore, inadequate $O_2$ supply to nerve tissue in an organism and subsequent oxidative stress caused by reperfusion can have detrimental consequences manifested at a systemic level. Thus, dealing with a reduced/absent $O_2$ supply and oxidative stress during reperfusion are factors cells and organisms must face in order to maintain adequate performance and survival.

As a result of natural selective pressures, some vertebrate (Buck and Pamenter, 2006) and invertebrate (Azad and Haddad, 2009) species have developed molecular and physiological mechanisms to tolerate and cope with low or null $O_2$ levels for a prolonged amount of time (from hours to months) with no apparent harmful consequences. However, most mammals cannot tolerate hypoxia/anoxia without undergoing severe cellular damage or death (Hermes-Lima and Zenteno-Savín, 2002). Despite the importance $O_2$ has for proper cellular and organismal performance, we still do not have a complete understanding of the molecular and physiological processes that take place during hypoxia/anoxia or the strategies that protect tolerant species during the absence of $O_2$ and subsequent reperfusion. Understanding such processes will not only improve our knowledge on the subject but also allow development of therapeutic treatments aimed at reducing cellular damage caused by physiological disruptions that limit $O_2$ availability in a constant or intermittent fashion. Consequently, detrimental effects of disruptive events related to constant hypoxia (e.g. asthma, ischaemia and traumatic brain injury) and intermittent hypoxia (e.g. sleep apnoea, central hypoventilation syndrome and intermittent vascular occlusion in sickle cell anaemia) could be treated and lessened (Azad et al., 2009).

*Drosophila melanogaster* is a remarkably interesting and promising study model given its tolerance to anoxia (Krishnan et al., 1997) and the power of its genetic and molecular tools (Azad and Haddad, 2009). Additionally, genes have been mostly conserved from *D. melanogaster* to humans through evolution (Haddad and Ma, 2001). In fact, the fly shares 65–70% of the disease genes present in humans (Azad et al., 2009) and it has been useful in establishing the relationship of these genes to particular diseases (Fortini et al., 2000). Furthermore, an increasing number of studies have used *D. melanogaster* as a model organism of brain diseases (e.g. Parkinson's disease, Alzheimer's disease) and central nervous system injury (Jeibmann and Paulus, 2009). Consequently, these features allow characterization of physiological and molecular mechanisms involved in the response to and tolerance of anoxia, and comparison with the mechanisms present in other animals. Ultimately, common response targets can be identified and manipulated, and this could be used in the development of therapeutic treatments to relieve the side effects of disruptive events like stroke or heart attack.

In mammals, spreading depression (SD) is a phenomenon comprising a substantial relocation of ions between intracellular and extracellular compartments; additionally, it coincides with the propagation through grey matter of a nearly complete brain cell depolarization (Somjen, 2001). Generally a benign phenomenon, SD can be elicited by mitochondrial blockers, inhibition of $Na^+/K^+$-ATPase, simulated ischaemia, KCl application and hyperthermia (Rodgers et al., 2010). Furthermore, it has been associated with a

rise in extracellular potassium concentration ($[K^+]_o$), which is cleared when the stressor is removed (Rodgers et al., 2007). However, repetitive SD in the penumbra (healthy tissue around a brain infarct) further stresses the tissue, generally producing cell swelling (Andrew and MacVicar, 1994), a stable elevated $[K^+]_o$ (Branston et al., 1977), dendritic beading (Obeidat et al., 2000) and necrosis. Mammalian SD shares many characteristics with SD events elicited in the metathoracic ganglion of the migratory locust (*Locusta migratoria*) (Rodgers et al., 2010). Moreover, studies in the locust have used $[K^+]_o$ as a way to assess neural failure in this ganglion during SD repetition (Armstrong et al., 2009; Rodgers-Garlick et al., 2011). Likewise, it is possible that anoxia elicits SD-like events in *D. melanogaster* brain (Armstrong et al., 2011) with an eventual ion redistribution and cell membrane depolarization. Consequently, assessment of brain $[K^+]_o$ can be used to evaluate the integrity of the fruit fly's brain physiology while reperfusion damage is inflicted by repetitive anoxia. The inability of the *D. melanogaster* brain to reach the initial $[K^+]_o$ baseline after repeated anoxic depolarizations (ADs) is reminiscent of the disruption observed in the mammalian penumbra, evidenced as a sustained increment in $[K^+]_o$ baseline (Armstrong et al., 2011).

Changes in temperature affect the rate of different biochemical processes. This phenomenon can be expressed as a $Q_{10}$ factor, which corresponds to the rate change caused by a temperature increase of 10°C (Robertson and Money, 2012). *Drosophila melanogaster*'s metabolic rate (MR) can be easily manipulated by changing the temperature of its surroundings. This permits assessment of MR effects on neural failure during repeated reperfusion. Provided that the fruit fly's MR has a $Q_{10}$ of 2.2 (Schilman et al., 2011), temperature is expected to affect damage and recovery rates during anoxia and reperfusion. In the course of anoxia, low temperature probably decreases the build-up of anaerobic metabolites, like alanine, acetate and lactate (Feala et al., 2007), and possibly delays the consumption of endogenous antioxidant enzymes and energy metabolites (Zhang et al., 2011). When $O_2$ supply is restored, hypothermia could also reduce the build-up of reactive oxygen/nitrogen species (ROS/RNS) and slow down recovery and repair processes (e.g. ATP production, enzymatic repair). Furthermore, it can interfere with apoptosis activation by targeting different steps in the pathways (Liu and Yenari, 2007; Liu et al., 2008) and can upregulate the expression of trophic factors involved in cell survival and growth (Yenari and Han, 2012). The combination of these effects probably underlies the protective nature of low temperatures during metabolic challenge. Despite the fact that the mechanisms of hypothermia protection are slowly beginning to be understood, mounting evidence in mammalian models shows its role in the therapeutic treatment of brain and spinal cord trauma, and experimental stroke (Yenari and Han, 2012). Although these models have shown that a decrease in temperature reduces ischaemic injury damage, glutamate release and free radical production (Busto et al., 1987; Globus et al., 1995; Huh et al., 2000), clinical trials have been hindered by issues such as reducing body temperature while avoiding potentially detrimental effects of cooling, and establishing an effective time window for hypothermia application (Yenari and Han, 2012). However, clinical implementation of low temperature therapy has proven beneficial after traumatic brain injury (Marion et al., 1997), anoxic brain injury caused by cardiac arrest (Bernard et al., 2002) and hypoxic ischaemic neonatal encephalopathy (Gluckman et al., 2005).

The anoxia/hypoxia response in *D. melanogaster* is paradigm dependent (Liu et al., 2006; Azad et al., 2009). So far, studies have mainly focused on the effects of constant hypoxia/anoxia (Krishnan et al., 1997; Le Corronc et al., 1999; Liu et al., 2006; Lighton, 2007; Schilman et al., 2011) and just a few have considered the fruit fly's response under a repetitive protocol (Lighton and Schilman, 2007; Azad et al., 2009; Armstrong et al., 2011). Furthermore, only Armstrong and colleagues (Armstrong et al., 2011) have used brain $[K^+]_o$ as a way of assessing neural failure during repetitive anoxia. Consequently, our knowledge of the mechanisms involved in the fruit fly's responses to anoxia iteration is at an early stage and needs to be increased. Repeated anoxia disrupts brain $K^+$ homeostasis in the fruit fly, causing an increment in $[K^+]_o$ baseline (Armstrong et al., 2011). However, it is not clear (1) what kind of anoxia/normoxia pattern is more disruptive, and (2) how temperature can modulate brain $K^+$ homeostasis disruption. The present study addressed these questions by measuring brain $[K^+]_o$ in male *D. melanogaster* (w1118) individuals at three different temperature levels (cold, 17°C; room, 23°C; warm, 29°C) during AD iteration.

## MATERIALS AND METHODS

### Animals

Male adult *D. melanogaster* Meigen individuals (w1118, 4–6 days old after emergence from pupal stage) were used. Flies were kept under a 12 h:12 h light/dark photoperiod in the Biosciences Complex at Queen's University. Room temperature was 22.0±0.25°C. The animals were raised on standard medium (see Mileva-Seitz et al., 2008): 0.01% molasses, 8.20% cornmeal, 3.40% killed yeast, 0.94% agar, 0.18% benzoic acid, 0.66% propionic acid and 86.61% water. They were chosen without using any selection criteria before every experiment. A total of 3–4 male flies were assessed per treatment.

### Preparation of $K^+$-sensitive microelectrodes

Potassium-sensitive microelectrodes were prepared as described elsewhere (Rodgers et al., 2007). All chemical substances were obtained from Sigma-Aldrich (Oakville, ON, Canada). Unfilamented glass capillary tubes with a diameter of 1 mm (World Precision Instruments, Sarasota, FL, USA) were washed with methanol (99.9%) and dried on a hot plate before being pulled to form a low-resistance tip (5–7 MΩ). Subsequently, they were made hydrophobic by application of dichlorodimethylsilane (99%) on a hot plate (100°C) for 1 h. The tip of each electrode was filled with potassium ionophore I-cocktail B (5% valinomycin) to create an artificial $K^+$-selective membrane; then the electrodes were backfilled with a 500 mmol $l^{-1}$ KCl solution and suspended in distilled water until needed in an experiment. Reference electrodes with a 5–7 MΩ resistance tip were made using glass filamented capillary tubes (1 mm in diameter) and backfilled with 3 mol $l^{-1}$ KCl before the beginning of an experiment.

### Preparation and setup

Flies were held using a fine-tip aspirator and were immobilized on a wax block (2×4×4mm) using minuten pins without application of anaesthesia. A chlorided silver wire was inserted between the fourth and fifth abdominal terga to ground the preparation (Fig. 1A). With a pair of microscissors, a small window (0.06×0.02 mm) was opened behind the ocelli, and brain extracellular potassium voltage was measured continuously by means of a $K^+$-sensitive microelectrode. Taking into account the location of the microelectrode, it was possible to establish likely neuropile regions whose $[K^+]_o$ was assessed during the experiments: the superior protocerebrum (medial and lateral), a region of unnamed neuropile, and the fan-shaped body in the central body complex. ADs were generated by repetitive anoxic comas induced through application of compressed pure nitrogen gas (>99%, 4 l $min^{-1}$) alternated with

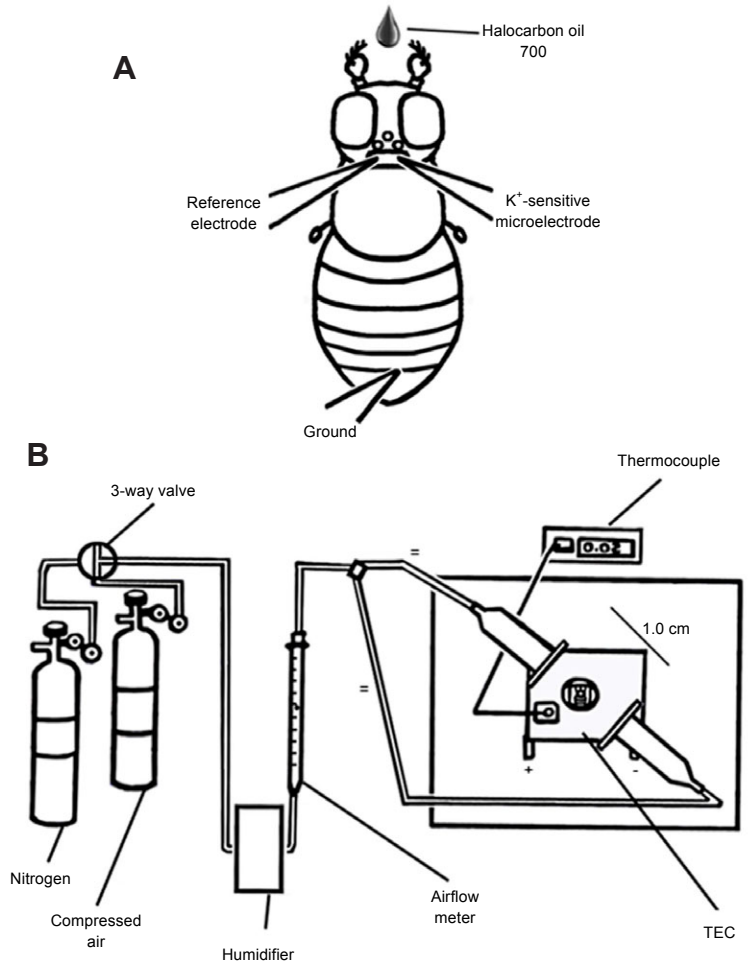


Fig. 1. Preparation and general setup. (A) A window (0.06×0.02 mm) was opened at the back of the head behind the ocelli. Reference and $K^+$-sensitive microelectrodes were introduced into the brain and $[K^+]_o$ was continuously measured. The preparation was grounded using a chlorided silver wire inserted between the fourth and fifth abdominal terga. Desiccation was avoided by sealing the head window with Halocarbon oil 700. (B) The immobilized fruit fly was placed on a thermoelectric cooler (TEC) between two 100 ml syringes connected to compressed air and $N_2$ tanks. A 3-way valve allowed alternation of the gases at a rate of 4 l $min^{-1}$ in a continuous way. The TEC temperature was constantly monitored with a thermocouple and the hydric integrity of the preparation was guaranteed by humidifying the gases in a flask with warm distilled water (humidifier).

periods of normoxia. During normoxia, compressed air (mixture of $O_2$, 19.5–23.5% and $N_2$, 76.5–80.5%) was applied at the same gas flow rate. Compressed air/$N_2$ tanks were obtained from Praxair (Mississauga, ON, Canada). The fruit fly was placed between two 100 ml syringes connected to a 3-way valve, allowing a separation between syringes of 1.0 cm (Fig. 1B). The valve permitted alternate application of the gases of interest in an uninterrupted way. Desiccation was prevented by sealing the head window with Halocarbon oil 700 (Halocarbon Products Corporation/Sigma-Aldrich) and passing the gases through an Erlenmeyer flask containing warm water (humidifier). Proper control of this variable was of great importance given that, during long anoxic periods (>60 min) or repeated reperfusion, *D. melanogaster* loses almost a quarter of its mass per hour owing to dehydration caused by loss of spiracular control (Lighton and Schilman, 2007).

### Oxygen concentration assessment

Oxygen concentration assessment of the experimental set-up during anoxia was performed using a DO 1200 dissolved oxygen sensor (Sensorex, Garden Grove, CA, USA). The probe was connected to a voltmeter, and different $O_2$ concentrations were expressed as a voltage reading (mV). Calibration was performed by insertion of the probe into an Erlenmeyer flask containing pure $N_2$ ($O_2$=0%), pure $O_2$ ($O_2$>99%) and compressed air ($O_2$=19.5–23.5%). During a $N_2$ pulse, subsequent $O_2$ content assessment of the gas between the syringes illustrated in Fig. 1B confirmed that the set-up was anoxic.

### Extracellular potassium recordings

Reference and $K^+$-sensitive microelectrodes were connected to a DUO773 two-channel intracellular/extracellular amplifier (World Precision Instruments) and calibration was performed at room temperature (22.0±0.25°C) using 15 mmol $l^{-1}$ KCl + 135 mmol $l^{-1}$ NaCl solution and 150 mmol $l^{-1}$ KCl solution. Theoretically, a 10-fold change in $K^+$ concentration should produce a voltage of ~58 mV, and only pairs of electrodes whose reading was 58±4 mV were selected for an experiment. Both electrodes were introduced into the brain through the window previously opened and $K^+$ voltage was continuously recorded. Brain extracellular potassium concentration values were obtained using the Nernst equation (Rodgers et al., 2007).

### Temperature variation and tissue/plate correlation

The wax block with the immobilized fruit fly was positioned on a plastic disc (1 mm thick and 5 mm in diameter) located on a thermoelectric cooler (TEC) (Fig. 1B). Nitrogen and compressed air were alternately applied while temperature was monitored by a thermocouple probe located on the TEC. Three temperature levels were tested: cold (17°C), room (23°C) and warm (29°C). The temperature treatments were established based on two criteria: firstly, it was necessary for the chosen temperatures to be found in the fruit fly's natural environment and secondly, the temperatures needed to be easily and consistently reached using the TEC available. The latter criterion constrained the possible range of temperatures to be tested, as tissue temperatures lower than 17°C were difficult to reach under constant gas flow.

In order to establish the fruit fly's internal temperature just by knowing the TEC temperature, male flies (10 for warm and 10 for low temperatures) were immobilized and placed in the set-up illustrated in Fig. 1B. One thermocouple probe was inserted in the fruit fly's head and a second one was attached to the TEC surface. Different increasing voltages (0.4 V/2 min) were applied to the TEC and the respective thoracic and plate temperatures were registered. A linear regression was performed by plotting mean head temperature *versus* mean TEC temperature ($r^2$=0.99). This established the following correlations: 17°C (head)=–1.0°C (TEC); 23°C (head)=19.6°C (TEC); 29°C (head)=40.3°C (TEC).

### Nitrogen-delivery pattern and variables

Repeated ADs were elicited by $N_2$-induced anoxic comas (3.0 min each) alternated with periods of normoxia (0.5 min each) for 30 cycles. Each anoxic bout was associated with an abrupt surge in $[K^+]_o$, which returned to baseline during normoxia (Fig. 2A). The presence of anoxic $K^+$ surges and the inability to return to the initial $[K^+]_o$ baseline during $O_2$ re-establishment was taken to indicate a disruption of $[K^+]_o$ homeostasis caused by the iteration of ADs. The pattern used was identified as the most disruptive in a pilot study that assessed the effect of anoxia/normoxia frequency and duration on brain $[K^+]_o$ maintenance through 17 different $N_2$-delivery patterns (data not shown). Two types of control were implemented: the first one involved continuous compressed air flow on the fruit fly for the whole duration of the experiment, except for an anoxic coma elicited at the beginning and at the end (air control, performed at all temperatures); the second one was based on a 90 min $N_2$ pulse followed by compressed air during the remaining experiment time ($N_2$ control, performed only at room temperature).

The response variables analysed included time to surge ($t_{surge}$), $[K^+]_o$ baseline before surge, surge amplitude, and time to recovery ($t_{recovery}$) (Fig. 2B). The parameter $t_{surge}$ was defined, in the unconverted $K^+$-voltage trace, as the time taken by the system to show a surge after $N_2$ was applied. Furthermore, the beginning of a surge was considered as the point in this trace where there was a sustained increase of at least 1 mV after $N_2$ application. The variable $[K^+]_o$ before surge was the lowest $[K^+]_o$ reading before a surge was present. The derived variable total $[K^+]_o$ baseline variation ($\Delta[K^+]_o$) was defined as the difference between $[K^+]_o$ baseline before the last and the first surges of the trace. Surge amplitude was the $[K^+]_o$ difference between the point of a surge where $N_2$ was turned off and the lowest point of the trace before the surge. The variable $t_{recovery}$ was defined as the time taken by the system to reach a stable $[K^+]_o$ baseline after $N_2$ was turned off at the end of an experiment. The parameter POR (percentage of recovery) expressed the amplitude of surges 2–30 as a percentage of surge 1, which was expected to have the highest amplitude. The derived variable ΔPOR was calculated by subtraction of POR for the last surge of the trace from POR from the first surge of the trace. As there were a large number of cycles applied per experiment (30), only the odd surges were used to obtain data points for all the variables.

### Statistical analyses

Data were plotted and analysed using SigmaPlot 11.0 (Systat Software Inc., Chicago, IL, USA). The values reported correspond to the means ± s.e.m. Variables were analysed using one-way ANOVA, and *post hoc* comparisons were performed by the Holm–Sidak method. Logarithmic transformation of the data was performed when necessary in order to meet the variance assumption of parametric tests (i.e. homoscedasticity). The significance level

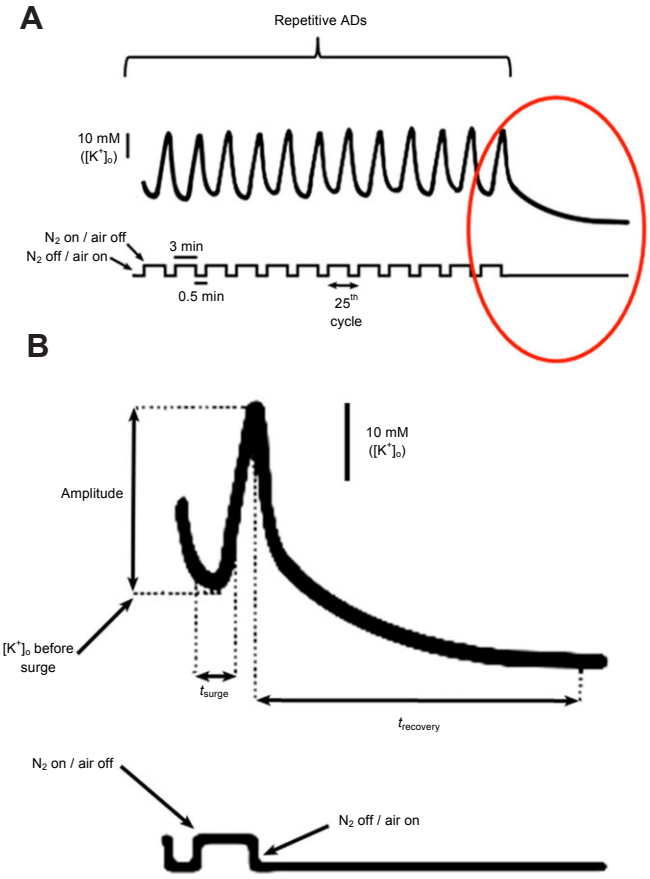


Fig. 2. Nitrogen-delivery pattern and variables. (A) The last 12 $[K^+]_o$ surges of a hypothetical cold temperature trace during repetitive anoxia. A 3 min/0.5 min anoxia/normoxia pattern was applied for 30 cycles (bottom trace). $N_2$-induced anoxic comas were used to cause anoxic depolarizations (ADs) related to sudden $[K^+]_o$ surges in the brain (top trace). (B) Enlarged version of the area circled in A. Application of humidified $N_2$ (bottom trace) caused a disruption in $[K^+]_o$ homeostasis (top trace), and a return to normoxia using humidified compressed air prompted recovery of $[K^+]_o$ baseline. Surge amplitude, $[K^+]_o$ baseline before surge, time to surge ($t_{surge}$) and time to recovery ($t_{recovery}$, only after the last surge) were the response variables analysed.

set for all the analyses was $\alpha$=0.05. Figures show statistical groupings using letter designations: treatments with different lettering are significantly different ($P$<0.05).

## RESULTS

### Hypothermia increases $[K^+]_o$ baseline maintenance during AD repetition

Repeated $N_2$ pulses delivered to the flies caused anoxic comas related to a disturbance in ion homeostasis in the brain (AD). A resulting abrupt $[K^+]_o$ surge occurred every time the animals went into a coma, and $[K^+]_o$ returned to baseline when normoxia was restored. The iteration of induced ADs produced disruption of $[K^+]_o$ homeostasis, which eventually was manifested as the temporary inability to maintain a stable baseline.

$[K^+]_o$ baseline was not disturbed throughout every experiment at 17°C (Fig. 3A). At 23°C, all flies recovered their initial baseline values after the experiments. However, baseline disruption was evident during AD repetition (Fig. 3B). In contrast, flies at 29°C never recovered after the AD pattern was applied (Fig. 3C). The

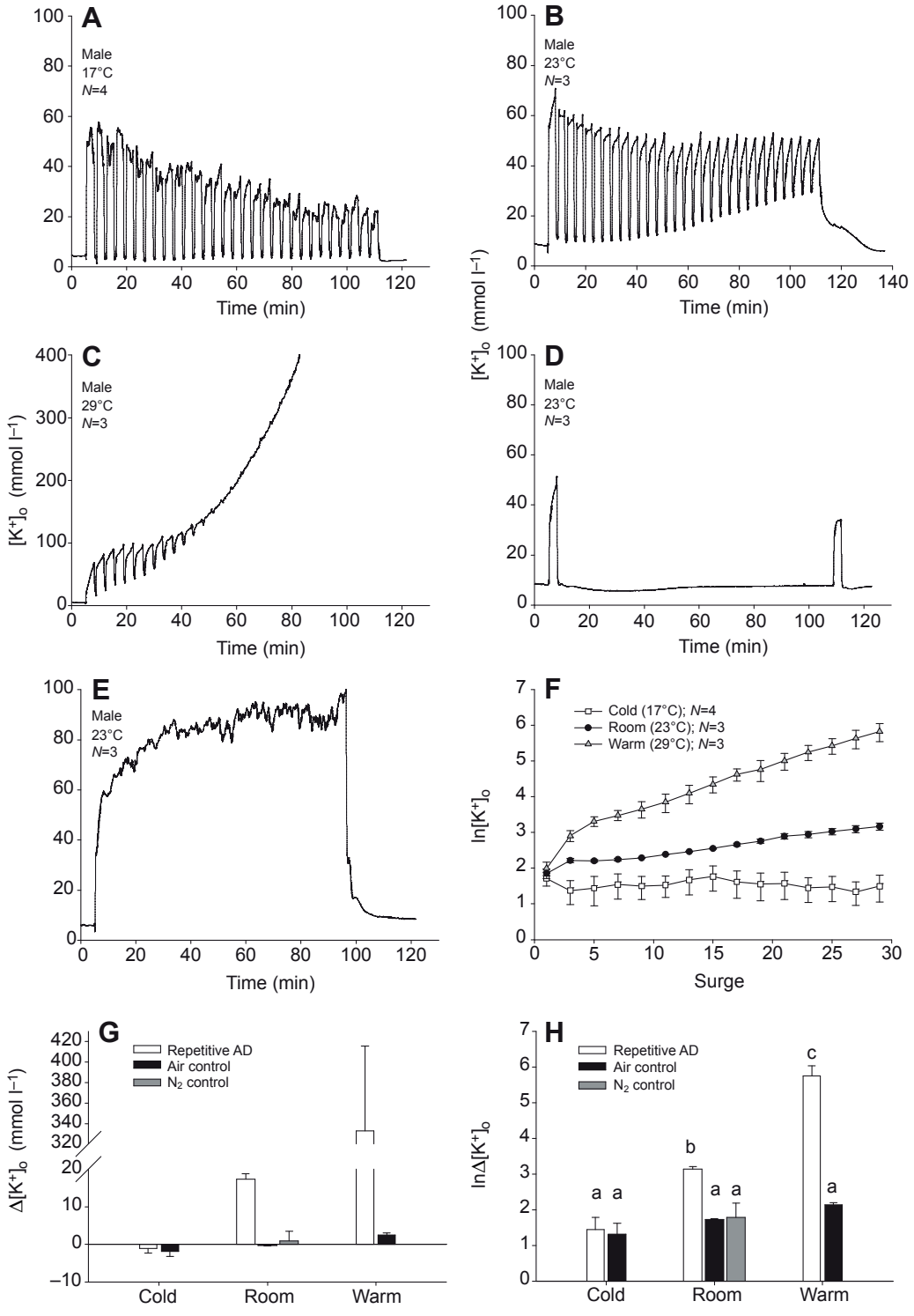


Fig. 3. Effect of temperature on male $[K^+]_o$ maintenance during repetitive ADs. (A) Low temperature (17°C) prevented repetitive ADs from producing $[K^+]_o$ baseline disruption. After the 30th surge, $[K^+]_o$ returned in about 3 min to values close to the initial baseline. (B) At 23°C, there was an increase in $[K^+]_o$ baseline caused by the iteration of ADs. When AD repetition ceased, baseline returned to values similar to those recorded before the 1st surge in ~20 min. (C) Warm temperature (29°C) severely exacerbated the loss of $[K^+]_o$ homeostasis, and baseline reached extremely high values. Flies never recovered, and the second half (approximately) of the surges had very small amplitudes. The *y*-axis was reduced in order to show a range of possible physiological concentrations. Higher $[K^+]_o$ values were obtained but were considered artifactual. (D) Absence of AD repetition (air control) did not cause any obvious baseline change. (E) Control experiments consisting of 90 min of anoxia and 15 min of normoxia during one cycle ($N_2$ control) did not cause any relevant $[K^+]_o$ increment either (performed only at room temperature). (F) The ln-transformed response variable ($\ln[K^+]_o$) showed three distinct trends in $[K^+]_o$ maintenance: hypothermia stabilized baseline values, hyperthermia severely exacerbated the loss of $[K^+]_o$ homeostasis, and room temperature produced intermediate values. (G) Mean $\Delta[K^+]_o$ per temperature supported the trends evident in F and suggested that controls had no substantial effect on $[K^+]_o$ baseline disruption. (H) Statistical analyses on $\ln\Delta[K^+]_o$ showed significant differences among the three treatments ($P<0.001$ for all tests) and, except for low temperature ($P=0.721$), between the treatments and their respective controls ($P\leq0.003$ for all tests) (one-way ANOVA, pairwise multiple comparisons performed by the Holm–Sidak method). For F–H, values are means ± s.e.m., $N=3$ for all treatments (except for $N_{cold}=4$) and controls, and different lowercase letters indicate different statistical groupings.

concentrations observed were excessively high and only a subset of physiologically possible values was considered. In order to assess the influence of the preparation itself on $[K^+]_o$ baseline at every temperature, experiments lacking AD iteration were performed (air controls). There was no evident brain $K^+$ homeostasis disruption (Fig. 3D). Likewise, the extra set of controls consisting of 90 min of anoxia and 15 min of normoxia ($N_2$ controls) did not cause any apparent permanent increment above baseline at room temperature (Fig. 3E). Mean $\ln[K^+]_o$ before surges 1–29 (only the odd surges were analyzed) showed three very clear trends during AD iteration: hypothermia stabilized $[K^+]_o$ baseline, hyperthermia caused a marked baseline increase, and room temperature produced intermediate values (Fig. 3F). The logarithmic transformation of the data was necessary given the high baseline increment produced by AD repetition at warm temperature.

Considering the untransformed response variable, hypothermia caused a slight $\Delta[K^+]_o$ decrease ($-1.1\pm1.3$ mmol $l^{-1}$), while severe ($332.7\pm83.0$ mmol $l^{-1}$) and moderate ($17.3\pm1.4$ mmol $l^{-1}$)

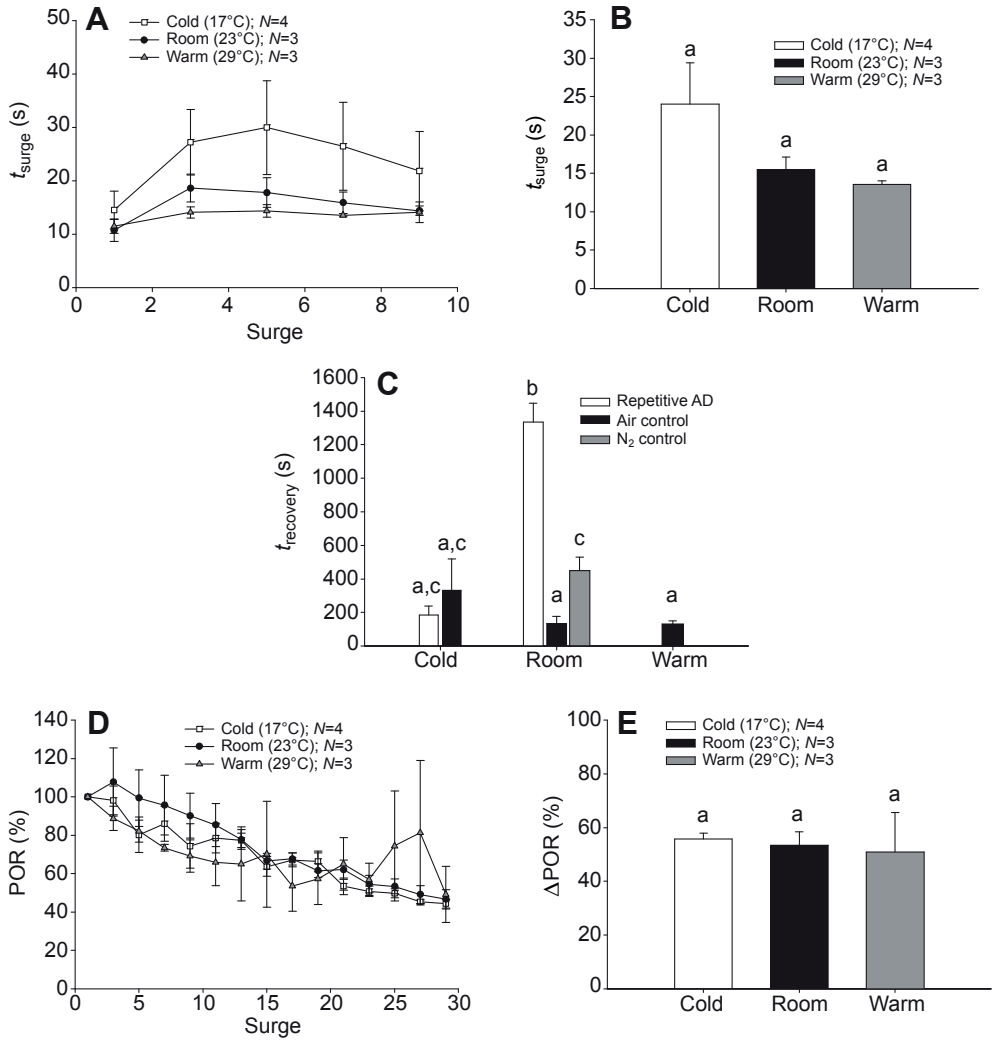


Fig. 4. Influence of temperature on $t_{surge}$, $t_{recovery}$ and percentage of recovery (POR) in males, during repetitive ADs. (A) Comparison of $t_{surge}$ for the first nine surges (only odd surges were considered) did not show obvious differences between temperatures. (B) Mean $t_{surge}$ supports this. An ANOVA (one-way) reported no significant differences between the treatments ($P$=0.194). (C) Mean $t_{recovery}$ was significantly higher at room temperature than during hypothermia and for controls (one-way ANOVA with Holm–Sidak method as pairwise multiple comparison test, $P$<0.001 for all tests). There were no statistically significant differences between controls and the cold temperature treatment ($P$>0.05 for all tests). A 90 min $N_2$ pulse followed by 15 min of normoxia ($N_2$ control) caused higher $t_{recovery}$ than a lack of AD iteration (air control) at 23 and 29°C ($P$=0.038 for both tests) and was not significantly different from the low temperature treatment ($P$=0.059) and air control at 17°C ($P$=0.402). (D) There was a gradual POR reduction in every treatment as recurrent ADs were applied, suggesting no effect of temperature on the variable. (E) Mean ΔPOR and a one-way ANOVA ($P$=0.910) reported no differences between treatments. For all plots values are means ± s.e.m., $N$=3 for all treatments (except for $N_{cold}$=4) and controls, and different lowercase letters indicate different statistical groupings.

increments were observed at warm and room temperature, respectively (Fig. 3G). At 17°C and 23°C, air controls caused a minor $\Delta[K^+]_o$ reduction (–1.9±1.3 and –0.3±0.1 mmol $l^{-1}$, respectively), whereas at 29°C they showed a small increase (2.5±0.5 mmol $l^{-1}$). Likewise, $N_2$ controls slightly increased $\Delta[K^+]_o$ at room temperature (1.0±2.6 mmol $l^{-1}$). A one-way ANOVA carried out on the $\ln\Delta[K^+]_o$ data (Holm–Sidak method as *post hoc* multiple comparison test) showed statistical differences between all temperatures ($P$<0.001 for all tests; Fig. 3H). The low temperature treatment was statistically grouped with its respective air control ($P$=0.721). In contrast, room and warm temperatures were significantly different from their corresponding controls ($P$<0.003 for all tests). Comparison of all controls showed no statistical differences ($P$>0.051).

### Temperature does not significantly affect $t_{surge}$

At 17 and 23°C, $t_{surge}$ traces were constant and did not show marked variation. However, at 29°C, surge amplitude became gradually and considerably smaller after the 13th surge until the point of being almost imperceptible (Fig. 3C). This caused a substantial increase in $t_{surge}$ as it took more time for every surge to cause a sustained rise of 1 mV after $N_2$ application (see Materials and methods for the definition of $t_{surge}$). Thus, for the sake of comparison, only those surges that had a similar shape between the temperatures were considered, resulting in an analysis focused on the first nine surges (only odd surges were sampled). Traces for all temperatures were not clearly separated (Fig. 4A). A one-way ANOVA carried out on mean $t_{surge}$ reported no differences between the treatments ($P$=0.194, Fig. 4B).

### AD iteration increases $t_{recovery}$ at room temperature

At 17 and 23°C, fruit flies regained their initial $[K^+]_o$ baseline after the 30th surge (Fig. 3A,B). Conversely, 29°C had a severe disruptive effect on $[K^+]_o$ homeostasis and flies never recovered (Fig. 3C). Additionally, $t_{recovery}$ depended on the temperature applied, as flies at 17°C experienced faster baseline recuperation, whereas flies at 23°C recovered in two distinct phases: an initial fast stage and a subsequent slow stage. Therefore, flies at room temperature took more time to regain their initial baseline. A one-way ANOVA (pairwise multiple comparisons performed by the Holm–Sidak method) showed that $t_{recovery}$ at 23°C (1335.8±112.3 s) significantly increased compared with that for hypothermia (184.4±53.0 s) and controls performed at all temperatures (cold, air control $t_{recovery}$=332.1±186.5 s; room, air control $t_{recovery}$=134.1±43.9 s; room, $N_2$ control $t_{recovery}$=451.1±79.3 s; warm, air control $t_{recovery}$=132.8±17.4 s; $P$<0.001 for all tests; Fig. 4C). The low temperature treatment was statistically grouped with all controls, regardless of temperature ($P$>0.059 for all tests). No dissimilarities were found between controls except for $N_2$ controls, which were different from air controls at 23°C and 29°C ($P$=0.038 for both tests).

### AD repetition decreases POR independently of temperature

In order to make surge amplitude comparable between treatments, amplitudes for surges 2–29 (only odd surges were considered) were expressed as a percentage of the first surge (POR). There were no differences between cold, warm and room temperature data, and a gradual decrease in amplitude was evident as more ADs were delivered (Fig. 4D). Calculation of ΔPOR corroborated a reduction in POR at 17°C (55.7±2.2%), 23°C (53.4±5.0%) and 29°C (50.8±14.7%) (Fig. 4E). Additionally, the lack of differences between treatments was supported by a one-way ANOVA ($P$=0.910).

## DISCUSSION

The present study focused on how $K^+$ homeostasis can be affected by repeated $O_2$ reperfusion in the fruit fly's brain neuropile. Additionally, temperature and its modulating effects on possible oxidative stress were also investigated. As only one known study has monitored brain $[K^+]_o$ in *D. melanogaster* during AD iteration (Armstrong et al., 2011), and given that it is still not clear what mechanisms allow the fruit fly to tolerate repeated $O_2$ reperfusion, it is important to broaden our knowledge in this field by testing additional variables (e.g. temperature). Based on our findings, we conclude that (1) reperfusion patterns consisting of long anoxia, short normoxia and high cycle frequency increase disruption of brain $[K^+]_o$ baseline maintenance, and (2) hypothermia has a protective effect on brain $K^+$ homeostasis during repetitive anoxia.

### $t_{surge}$

Lack of ATP during anoxia causes failure of the $Na^+/K^+$ pump and membrane depolarization; consequently, $K^+$ flows out of the cell possibly through different types of $K^+$ ion channels (Ransom and Philbin, 1992). Given that the $Q_{10}$ value for ion channel currents is approximately equal to 2 (Buck and Pamenter, 2006) and that the MR of *D. melanogaster* has a $Q_{10}$ value of 2.2 (Schilman et al., 2011), temperature was expected to modulate $t_{surge}$ in an inversely proportional fashion during AD iteration. However, this trend was not evident in the data collected (Fig. 4A). Furthermore, there was no significant difference between the treatments (Fig. 4B). Interestingly, a temperature decrease of 12°C approximately doubled the $t_{surge}$ values (from 13.5±0.5 to 23.9±5.4 s), coinciding with what the $Q_{10}$ values would predict. Nonetheless, we believe that these results must be interpreted with caution given the size of the collected samples.

AD iteration had no apparent effect on the flies, and traces at 17 and 23°C remained approximately unchanged after every reperfusion considered (Fig. 3A), even after 30 anoxia/normoxia cycles (data not shown). Taking $t_{surge}$ as an indirect measure of MR, the previous result suggests that AD iteration had no effect on MR. This also indicates that mechanisms responsible for anoxia-driven inactivation of $K^+$ ion channels (Hochachka, 1986; Buck and Pamenter, 2006) may have not been involved.

### $t_{recovery}$

Baseline recovery depends on clearance mechanisms, which may be mainly affected by temperature, ROS/RNS damage accumulation and ATP depletion. In the particular case of $K^+$, the $Na^+/K^+$-ATPase is a fundamental contributor to maintaining and re-establishing differential $K^+$ concentrations across the cell membrane (Ransom and Philbin, 1992). Furthermore, glial cells can also be responsible for removing the excess $K^+$ in the extracellular space, a process known as '$K^+$ siphoning' (Orkand et al., 1966), and $K_{ir}$ channels seem to be fundamental to this role in $[K^+]_o$ regulation. These channels are open at hyperpolarized membrane potentials and their $K^+$ conductance decreases with depolarization (Hibino et al., 2010). At room temperature, $t_{recovery}$ after AD repetition considerably increased compared with that in hypothermia and controls (air and $N_2$) (Fig. 4C), probably because damage rates were slightly higher than recovery rates. Therefore, repair mechanisms required more time to re-establish the initial $[K^+]_o$. The intermediate damage accumulated allowed identification of two stages during recovery; namely, a fast phase and a slow phase (Fig. 3B). We hypothesize that the fast phase was possibly carried out in greater part by the $Na^+/K^+$ pump in neurons and glial cells, and that subsequent hyperpolarization of cell membranes probably opened $K_{ir}$ channels in glial cells, allowing '$K^+$ siphoning' to perform most of the $K^+$ removal during the slow phase. Considering that recovery time from anoxia depends on the duration of the anoxic period (Krishnan et al., 1997; Lighton and Schilman, 2007), $N_2$ controls showed slightly greater $t_{recovery}$ values than air controls at room and warm temperature. In contrast, the lack of significant differences from controls and treatment at 17°C probably indicates a low temperature-driven reduction of the $Na^+/K^+$ pump activity. Finally, the absence of similarities between $N_2$ controls and treatment at 23°C demonstrates that the increase in $t_{recovery}$ evidenced at room temperature was caused by repetitive anoxia *per se*.

In contrast, hypothermia was protective and significantly reduced recovery time despite its possible slowing effect on the $Na^+/K^+$ pump activity. This suggests that low temperature decreased damage rates and permitted the fruit fly's brain to recover faster. Warm temperature possibly had the opposite effect and prevented flies from recovering after repeated ADs.

### Percentage of recovery

In all treatments, this variable was gradually reduced by AD repetition and the rate of decrease did not depend on temperature (Fig. 4D,E). A progressive fall in amplitude with each reperfusion underlay such POR decrement. At room temperature, amplitude reduction was the result of two aspects of the trace (Fig. 3B): a top component that caused decrement of the highest value reached by the surges, and a bottom component that produced an increment in baseline. We believe that the bottom component was the result of a failure of the clearance mechanisms (i.e. $Na^+/K^+$ pump and $K^+$ siphoning), and that the top component was probably underlain by progressive inactivation of accumulation mechanisms (i.e. $K^+$ ion channels) that restricted the outflow of $K^+$ and caused a gradual reduction in the maximum amplitude of the surges. Performance of clearance mechanisms could also have been affected by hypoxia-induced $Na^+/K^+$-ATPase endocytosis mediated by AMP-activated protein kinase (AMPK), a strategy already described in rat alveolar epithelial cells (Gusarova et al., 2009). Additionally, AMPK has been found to inhibit background $K^+$ channels in transfected HEK293 cells during hypoxia/anoxia (Kréneisz et al., 2009) and a calmodulin-mediated ion channel arrest model has previously been described in vertebrates (Buck and Pamenter, 2006).

The protective properties of hypothermia were evident during AD iteration: only the top component was conspicuous, suggesting activation of channel arrest mechanisms in order to reduce energy consumption by ion pumping (Hochachka, 1986) (Fig. 3A). Furthermore, the bottom component was absent, probably owing to optimal action of clearance mechanisms. In contrast, the bottom component was extremely dominant during hyperthermia and overrode the action of the top component (Fig. 3C). As cell

deterioration was possibly much greater than the recovery rate, $[K^+]_o$ baseline was rapidly and permanently lost.

### Temperature effect on $[K^+]_o$ maintenance during AD repetition

During AD iteration, hypothermia preserved the integrity of $K^+$ homeostasis (Fig. 3A). Low temperature affects transport mechanisms involved in ion balance (Carpenter, 1981), causing cell depolarization. However, *D. melanogaster* can maintain resting potentials during hypothermia better than other species (e.g. *Apis mellifera*), and only temperatures close to chill coma (7±0.9°C) cause $[K^+]_o$ increment (Hosler et al., 2000). Therefore, the temperature applied during the present study (17°C) may have slowed the $Na^+/K^+$ pump but did not affect the $K^+$ gradient maintenance. Evidence of this was the stable $[K^+]_o$ baseline recorded for 5 min before every experiment and for air controls, at low temperatures. During hypothermia, recovery rates were probably equal to or higher than reperfusion-driven damage rates, thereby causing increased tolerance to AD iteration. Actually, low temperature highly stabilized $[K^+]_o$ baseline and caused a slight decrease in $\Delta[K^+]_o$. The reduction was probably caused by solvent increment in the extracellular space produced by diffusion of water vapour from the environment to the tracheal system through the open spiracles.

At warm temperature, recordings showed a steady baseline before the beginning of the experiments and during air controls, suggesting optimal performance of ion pumps. Nevertheless, repeated reperfusion severely exacerbated $[K^+]_o$ baseline disruption in the fruit fly's brain. The values obtained were extremely high and only a subset of them, within a range of physiologically possible concentrations, was used for analysis. The range of acceptable values was established based on the intracellular potassium concentration ($[K^+]_i$) in the axoplasm of the squid, reported to be 369 mmol $l^{-1}$ (Steinbach and Spiegelman, 1943). Although lower $[K^+]_i$ values have been found in honeybee drone photoreceptors (127 mmol $l^{-1}$) and retina glial cells (132 mmol $l^{-1}$) (Coles et al., 1986), we believe that cell swelling and a subsequent extracellular space reduction accounted for the high $[K^+]_o$ values obtained. Possible cell damage may have been irreparable and flies never recovered their initial $[K^+]_o$ baseline (Fig. 3C). Furthermore, during recovery at warm temperature, ATP demand increases but $O_2$ diffusivity is barely changed; thus, $O_2$ cannot be efficiently delivered in order to provide the $Na^+/K^+$ pump with enough energy to clear $[K^+]_o$ (Frazier et al., 2001; Lighton, 2007).

$[K^+]_o$ homeostasis was moderately altered by AD iteration at 23°C. Flies recovered their initial baseline after sustained reperfusion (Fig. 3B), requiring a drastic anoxia/normoxia pattern (3 min/0.5 min for 30 cycles) in order to undergo baseline disruption. Increased accumulation of cell damage and less time to recover explain why a pattern with long anoxia, short normoxia and increased cycle frequency affects baseline maintenance in the fruit fly's brain. Contrastingly, the contribution of anaerobic metabolites to $K^+$ homeostasis loss appeared to be non-significant as a 90 min anoxic coma only caused a baseline increase of 1.0±2.6 mmol $l^{-1}$ (Fig. 3G). The effect of starvation was minimal as it has been shown that there is no mortality in w1118 flies food deprived for 24 h (Van Voorhies, 2009). Nonetheless, ATP depletion may have been partially responsible for the deterioration of the $Na^+/K^+$ pump performance and the gradual loss of $[K^+]_o$ baseline during AD iteration.

Increasing evidence in mammalian models and clinical trials supports the importance of hypothermia as a therapeutic agent against the detrimental effects of ischaemia (for a review, see Yenari and Han, 2012). Additionally, the fact that low temperature increased *D. melanogaster*'s tolerance to reperfusion is an important finding that will allow future dissection of the molecular pathways that confer protection during hypothermia.

## LIST OF ABBREVIATIONS

| | |
|---|---|
| AD | anoxic depolarization |
| $[K^+]_i$ | intracellular potassium concentration |
| $[K^+]_o$ | extracellular potassium concentration |
| $\Delta[K^+]_o$ | total $[K^+]_o$ baseline variation |
| MR | metabolic rate |
| POR | percentage of recovery |
| ΔPOR | total percentage of recovery variation |
| RNS | reactive nitrogen species |
| ROS | reactive oxygen species |
| SD | spreading depression |
| TEC | thermoelectric cooler |
| $t_{recovery}$ | time to recovery |
| $t_{surge}$ | time to surge |

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