Glial Hsp70 Protects K⁺ Homeostasis in the Drosophila Brain during Repetitive Anoxic Depolarization

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Abstract

Neural tissue is particularly vulnerable to metabolic stress and loss of ion homeostasis. Repetitive stress generally leads to more permanent dysfunction but the mechanisms underlying this progression are poorly understood. We investigated the effects of energetic compromise in Drosophila by targeting the Na⁺/K⁺-ATPase. Acute ouabain treatment of intact flies resulted in subsequent repetitive comas that led to death and were associated with transient loss of K⁺ homeostasis in the brain. Heat shock pre-conditioned flies were resistant to ouabain treatment. To control the timing of repeated loss of ion homeostasis we subjected flies to repetitive anoxia while recording extracellular [K⁺] in the brain. We show that targeted expression of the chaperone protein Hsp70 in glial cells delays a permanent loss of ion homeostasis associated with repetitive anoxic stress and suggest that this is a useful model for investigating molecular mechanisms of neuroprotection.

Introduction

Neural function is critically dependent on maintaining cellular ion homeostasis which in turn is dependent on an adequate energy supply. Loss of ion homeostasis with consequent depolarization of neurons and glia occurs in response to anoxia (anoxic depolarization; AD) and in healthy tissue complete recovery is possible on return to normoxia within species-specific time limits. Transient loss of ion homeostasis can occur spontaneously and spread through healthy tissue resulting in depolarization (spreading depolarization) and cessation of electrical activity (spreading depression; SD). Whereas Drosophila is established as an excellent genetic model for investigating effects of anoxia in whole organisms [1,2,3,4] we know little about how metabolic stress affects ion homeostasis in the fly brain or about mechanisms that could protect brain function in this system.

SD in the mammalian cerebral cortex occurs as a substantial redistribution of ions between intracellular and extracellular compartments coinciding with a near complete depolarization of a sizable proportion of brain cells. The disturbance propagates spontaneously and spread through healthy tissue resulting in depolarization (spreading depolarization) and cessation of electrical activity (spreading depression; SD). Whereas Drosophila is established as an excellent genetic model for investigating effects of anoxia in whole organisms [1,2,3,4] we know little about how metabolic stress affects ion homeostasis in the fly brain or about mechanisms that could protect brain function in this system.

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Results

In mammals [25] and locusts [21,22,26,27] mimicking the effect of energetic compromise with the Na$^+$/K$^+$-ATPase inhibitor ouabain generates spontaneous SD similar to PID. To determine if a similar phenomenon could occur in the fly brain we exposed flies to volatilized ouabain (10 μl of 100 mM; see e.g.[28]) for 1 hour and then videotaped individual fly behaviour. A proportion of flies were noticeably affected by ouabain treatment (65%). Affected flies alternated between activity (walking/flying/grooming) and coma (motionless and unresponsive to touch) which began 78.2 ± 13.2 min following ouabain treatment (n = 23); (Video S1). Successive comas increased in duration and the duration of the last coma was significantly longer than the first three comas (Fig. 1A). Before failure, flies exhibited a median of 5 comas and the last coma was penultimate to death which was determined by examination 24 hrs after the treatment. Coinciding with the increase in coma duration was a decrease in the duration of the interval between comas. The last coma interval was significantly shorter than the first four intervals (Fig. 1B). HS-treated flies (n = 25) were resistant to ouabain treatment with no flies exhibiting comas during 6 hrs of observation. However, 24 hrs after treatment 4 of the 25 HS-preconditioned flies that had been exposed to ouabain were dead. Electrophysiological measurements of [K$^+$]$_o$ in the brains of flies (Fig. 1Ci) exhibiting repetitive ouabain-induced comas showed recurrent surges of [K$^+$]$_o$ from a baseline of 13.1 ± 2.8 mM. The small size of the brain precluded measurement of propagation however these events were similar to spontaneous SD-like events evoked by ouabain in locust ganglia (Rodgers et al. 2007). The preparation eventually lost the ability to maintain ion homeostasis and baseline [K$^+$]$_o$ increased, terminating at a plateau of 54.1 ± 13.9 mM (Paired t-test, P = 0.026; n = 12). There was no recovery within the duration of the experiment (Fig. 1Cii). Of 44 similar preparations only 27%

![Figure 1. Exposure to ouabain induces repetitive comas coinciding with SD in the fly brain.](https://www.plosone.org)
showed \([K^+]_o\) surges, whereas 32\% showed a gradually rising \([K^+]_o\), 18\% showed an elevated plateau \([K^+]_o\) and 23\% showed \([K^+]_o\) that remained at low concentrations. These data suggest that spontaneous disturbances in \(K^+\) homeostasis occur in the fly brain during mimicked energy compromise resulting from inhibition of \(Na^+/K^+\)ATPase activity. To test if exposure to anoxia resulted in a similar phenomenon we repeatedly exposed flies to nitrogen gas while measuring \([K^+]_o\) in the brain.

Passing 100 \% \(N_2\) gas over the preparation rapidly and reliably evoked a negative DC potential shift and a simultaneous surge in \([K^+]_o\) in the fly brain which recovered when the fly was returned to normoxia (Fig. 2A). During anoxia the DC potential near the site of the \(K^+\)-sensitive electrode initially increased by 6.5±0.6 mV before a sharp negative deflection of -23.0±1.34 mV (n = 4).

These changes in the DC potential resemble those associated with AD [29] and PID [30] in the mammalian brain suggesting increased neuronal firing followed by a rapid onset of cellular depolarization. Increasing the duration of 100\% \(N_2\) exposure from 5 s to 90 s demonstrated a threshold duration for evoking the \([K^+]_o\) surge. Recovery was characterized by two phases of \([K^+]_o\) clearance (n = 5) (Fig. 2B) and suggests the involvement of two clearance mechanisms or two tissue compartments (e.g.[31]). The ability to control the timing of multiple \([K^+]_o\) surges in the brain rapidly and precisely is attractive and it formed the basis for our subsequent experiments.

To assess the deleterious effect of repetitive anoxia we exposed animals to cyclical bouts of 100\% \(N_2\) gas (2.5 min on/4 min off) and measured \([K^+]_o\) in the fly brain for 85 minutes. Under these

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**Figure 2. Sample traces of \([K^+]_o\) surges in the fly brain during anoxia induced by exposure to \(N_2\) gas (A).** 100\% \(N_2\) gas was delivered through a porous polyethylene substrate. Flow timing and rate was controlled and the gas stream completely covered the animal. A single surge of \([K^+]_o\) in the brain in response to a pulse of \(N_2\) recorded simultaneously with the extracellular DC potential. Three phases of \([K^+]_o\) increase are evident (slow, fast and slow). (B) Surges of \([K^+]_o\) in response to pulses of \(N_2\) that increased in duration from 5 s to 90 s (traces overlaid and aligned at the start of anoxia). Note that in this preparation durations of \(N_2\) exposure up to 40 s resulted in small increases of \([K^+]_o\) that recovered quickly. Thereafter there was a surge of rapidly increasing \([K^+]_o\) whose amplitude was dependent on the duration of \(N_2\) exposure until it reached a plateau that is not illustrated in this figure.

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experimental conditions, repetitive anoxia eventually was associated with a loss of ion homeostasis indicated by a gradual increase of $[K^+]_o$, to a plateau from which we never observed the preparation to recover, though we cannot be certain that longer periods of recovery would be ineffective (Fig. 3A, Ci, Control). In flies preconditioned with HS we observed robust protection of potassium ion homeostasis in the brain. Firstly, these flies displayed a strong ability to recover $[K^+]_o$ to baseline levels between exposures of N2 gas whereas untreated flies displayed a poor ability to maintain low $[K^+]_o$ between bouts of anoxia (Fig. 3A,Ci). Secondly, we observed a significant increase in the latency to the $[K^+]_o$ surge during each exposure to anoxia in HS-treated flies (Fig. 3Cii). Thirdly, we observed a reduction in the peak $[K^+]_o$ during surges in HS-treated flies compared to control flies (Fig. 3Ciii). To examine the role of Hsp70 during HS preconditioning we repeated our heat shock protocol in flies

Figure 3. HS preconditioning stabilizes $K^+$ homeostasis. Flies were subjected to repetitive anoxia (100% N2 gas) in a 2.5 min on/4 min off cycle. In all experiments anoxia rapidly induced a surge in $[K^+]_o$. (A), Sample recordings of brain $[K^+]_o$ taken from one control fly (black) and one HS fly (grey). (B), Sample recordings of brain $[K^+]_o$ taken from one untreated fly lacking all 6 copies of the hsp70 gene (hsp70-null) (black) and one HS-treated hsp70-null fly (HS hsp70-null) (grey). (C), Effects of repetitive anoxia on baseline $[K^+]_o$ (i), time to surge (ii) and peak $[K^+]_o$ (iii) of sequential surges during repetitive exposures to anoxia in control and HS-treated flies. (D), Effects of repetitive anoxia on baseline $[K^+]_o$ (i), time to surge (ii) and peak $[K^+]_o$ (iii) of sequential during repetitive exposures to anoxia in hsp70-null and HS-treated hsp70-null flies. Note that HS-treatment protects the brain from effects of repetitive anoxia but these are exacerbated after HS-treatment in hsp70-null flies. Points represent three or more data points and are displayed as means ± standard error. Numbers in parentheses represent sample sizes and single and double asterisks represent significant differences within SD #s ($P<0.05$ and $P<0.01$ respectively). doi:10.1371/journal.pone.0028994.g003
lacking all 6 copies of the hsp70 gene [32] (HS hsp70-null). These flies were less able to maintain K⁺ homeostasis compared to untreated hsp70-null flies (Fig. 3D). In addition, HS-treated hsp70-null flies were more vulnerable to anoxia and had shorter latencies to the beginning of each surge in [K⁺]o (Fig. 3Dii). Interestingly, under non-HS conditions the hsp70-null genotype was associated with an increased stability of [K⁺]o, during repetitive anoxia and we suggest that this could be due to mechanisms compensating for the loss of Hsp70 during development, as described for flies deficient in heat shock factor [33]. There was no clear difference of peak [K⁺]o between HS-treated and untreated hsp70-null flies (Fig. 3Diii). These data strongly suggest that Hsp70 plays a role in conferring tolerance to repetitive anoxia in the fly brain, specifically by improving the ability of the fly brain to maintain K⁺ homeostasis and delay the onset of K⁺ surges during anoxia. To test this directly we used the GALA-UAS binary system for tissue-specific expression of Hsp70.

A UAS-hsp70 fly line carrying a single copy of the Drosophila hsp70Ab gene with a c-myc tag sequence constructed in our laboratory [34] was crossed with GAL4 enhancer trap lines Repo- (pan-glial) and Elav- (pan-neuronal) GAL4. Immunocytochemistry confirmed that Hsp70 was present in the cytoplasm of cells and widely distributed throughout all of the brain tissue of both Elav-Gal4:UAS-hsp70 and Repo-Gal4:UAS-hsp70 flies (Fig. 4A,B). We monitored [K⁺]o in Repo-Gal4+, Elav-Gal4+ and UAS-hsp70+ in addition to Elav-Gal4:UAS-hsp70 (Neuronal Hsp70) and Repo-Gal4:UAS-hsp70 (Glial Hsp70) flies during repetitive N₂ anoxia. The three control groups were found to be not significantly different from each other and for statistical reasons they were combined (Controls). Targeted expression of Hsp70 in glia helped to maintain K⁺ homeostasis between bouts of repetitive anoxia (Fig. 4Ci). The ability to maintain low [K⁺]o in flies expressing Hsp70 in neurons was not significantly different from controls (Fig. 4C). However, the latency to the [K⁺]o surge was significantly longer than controls in flies with Hsp70 expression targeted to glia or neurons (Fig. 4Cii). Lastly we found that flies expressing Hsp70 in either glia or neurons had reduced peak [K⁺]o during repetitive anoxia (Fig. 4Ciii). It is important to note that for measurements of time to surge and peak [K⁺]o, (Fig. 4Cii & iii) both neuronal and glial Hsp70 were effective in providing protection. This is in contrast to baseline [K⁺]o, which was stabilized by glial Hsp70 more effectively than by neuronal Hsp70.

Discussion

The utility of Drosophila for insight into vertebrate brain function is well-established [35] and the fly has long been used as a model system for the dissection of the genetic basis of tolerance and...
susceptibility to hypoxia [1,36]. Drosophila are considerably more tolerant to hypoxia than mammals and can survive several hours of exposure to anoxia [2]. Additionally, Drosophila has been proposed as a model for studying ischemia and reperfusion injury based on the deleterious effects of repetitive returns to normoxia (every 20 min for 60 s) during maintained anoxia [3]. The linear relationship between the number of reperfusion events and ensuing mitochondrial and neuromuscular failure is reminiscent of the linear relationship between number of PIDs and infarct volume in rats [37]. Mechanisms providing protection of Drosophila against hypoxia include upregulation of chaperone proteins. For example Hsp70 and Hsp23 confer protection against constant hypoxia (1.5% O2) and targeted expression (heart and regions of the brain) increases survival time [38]. However these Hsps had no protective effects against intermittent hypoxia (a 20 min cycle ranging between 4 min at 1% O2 and 4 min of 21% O2). Additionally, these previous studies monitored survival of the whole organism and there is almost no information on disturbances in the fly CNS during anoxic coma.

In this study we demonstrate that during anoxic coma the fly brain exhibits a brain disturbance that shares the essential characteristics of AD. Notably, a rapid rise in [K+]o during exposure to anoxia which returns to near baseline levels following return to normoxia. We also demonstrate that exposure to the Na+/K+-ATPase inhibitor ouabain generates repetitive and spontaneous comas in intact freely behaving flies. We attributed the occurrence of these comas to concurrent [K+]o surges resulting from compromised Na+/K+-ATPase activity. Previous work by our lab has documented the occurrence of this phenomenon in the migratory locust (Locusta migratoria) CNS during exposure to ouabain [21,22,39] and we have proposed a model for its occurrence [22,23]. We believe that the spontaneous and repetitive surges in [K+]o, occurring in the fly brain are the essential distinguishing features of PIDs and sought to investigate exclusively the deleterious effect of repetitive anoxia in the fly brain. To do this we exposed flies to repetitive anoxia (cyclical bouts of 100% N2 gas; 2.5 min on/4 min off) and measured [K+]o in the fly brain for 85 minutes. This duty cycle was chosen because it was similar to that of the spontaneous [K+]o surges we observed in flies pre-treated with ouabain. It was also within the range of frequencies for repetitive PID evident in the rat brain after middle cerebral artery occlusion and reperfusion [40] and for recurrent SD induced by a single stimulus in the brain of Familial Hemiplegic Migraine Type 1 mutant mice [41]. Successful anoxia-induced surges culminated in an inability to restore [K+]o to baseline concentrations resulting in an elevated [K+]o plateau from which we never observed the preparation to recover (Fig. 3A, C6), but we cannot be certain that longer periods of recovery would be ineffective at restoring baseline [K+]o. We hypothesise that the gradual increase in brain [K+]o, between bouts of anoxia reflects an increasing impairment in the ability of cells to clear [K+]o. Although anoxia-induced surges were observed in all HS-treated flies, protection against the elevated [K+]o plateau was evident. HS-treated flies were better at maintaining low [K+]o, between bouts of anoxia. This might be due to an improvement in the ability to re-sequester [K+]o, on return to normoxia and may also account for the delay in producing a surge in [K+]o (Fig 3C6). Previous work has documented that HS preconditioning speeds the rate of recovery of the [K+]o surge in locusts [21] and up-regulation of Hsp70, using transgenic mice [42,43] and virally-mediated gene transfections [44,45], reduces neural damage in experimental models of stroke. Moreover there is considerable interest in Hsp70 as a therapeutic target in numerous brain pathologies [46,47]. Conversely, flies lacking Hsp70 displayed a poor ability to maintain K+ homeostasis which was worsened by HS preconditioning. Additionally, HS-treated hsp-70 null flies displayed a poor ability to resist the surge in [K+]o (Fig 3 Dii). It should also be noted that hsp-70 null flies, without HS, displayed longer time to surge values when compared to HS-treated hsp-70 null flies, however time to surge in hsp-70 null flies was not significantly different from HS-treated W1118 flies. This might be due to mechanisms compensating for the loss of Hsp70 during development, as described for flies deficient in heat shock factor which show an attenuated production of Hsp70 following HS but still acquire upregulate thermotolerance [33].

In a final set of experiments we targeted over expression of Hsp70 in either neurons or glia and exposed flies to repetitive bouts of anoxia. Interestingly, flies over expressing Hsp70 in glia were better at maintaining low [K+]o between bouts of anoxia. However flies over expressing Hsp70 in glia or in neurons displayed a significant delay in the time to surge of [K+]o. These data suggest a testable hypothesis that glial mechanisms have a predominant role in long-term maintenance of ionic gradients under cellular stress (i.e. the vulnerability to repetitive anoxia) whereas both neuronal and glial mechanisms are involved in the acute response of neural tissue to anoxia (i.e the propensity to generate AD).

Much of the long-term disability associated with stroke in mammals is thought to result from an increase in infarct volume during the period following a vascular accident. This increase is associated with PIDs that occur spontaneously and repetitively in the penumbral zone of vulnerable grey matter around the infarct. In spite of the critical role of events in the peri-infarct zone there is little understanding of how PIDs contribute to damage and consequently no treatments target PIDs. We suggest that repetitive anoxia in the Drosophila brain can serve as a model for PIDs and here we show that targeted expression of the chaperone protein Hsp70 in glial cells delays the loss of ion homeostasis associated with repetitive AD. We propose that this model of PID in Drosophila affords the opportunity to investigate many of the mechanisms of stroke injury using the powerful and rapid molecular genetic techniques available for the fly.

Materials and Methods

Flies lines
All experiments were performed at the Department of Biology at Queen’s University or at the Department of Biological Sciences at Florida Atlantic University. Flies 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 in 60-70 % humidity and reared in a 12 h/12 h (light/dark) cycle with lights on at 0800 h. Flies were maintained at equal densities (approximately 20 flies) in 30 ml plastic vials containing 5 ml of medium.

All electrophysiological experiments were performed on male flies aged 3-7 days post eclosion or older male flies (14-21 days). The w[1118]; P[w/+mC]=GAL4repo/TM3,Sb[y] and the w[+]; P[w/+mC]=GAL4-elav,L3 GAL4 enhancer-trap strains were obtained from the Bloomington Drosophila stock center and the UAS Hsp70 lines were constructed in our laboratory [34]. The hsp-70-null flies were obtained from Kent Golic [32]. Tissue-specific expression of Hsp70 was achieved using the Gal4/UAS system to drive Hsp70 expression in neurons (Elav-GAL4) or glia (Repo-GAL4). Flies overexpressing Hsp70 were obtained by crossing females carrying the UAS-hsp70-myc construct with males carrying a Gal4 transgene. To exclude effects due to heterosis, three controls were used: a UAS-hsp70/alone control, an Elav-GAL4/
alone, and a Repo-GAL4/alone control obtained by crossing, respectively, the UAS strain and the GAL4 strains with w^{1118}.

**Heat Shock**

Vials containing adult flies (3-7 days post eclosion) and medium were placed in a humid (~100 %) incubator at 36 °C for 1 h and subsequently removed to allow recovery at room temperature for 1 h.

**Electrophysiology**

To facilitate handling, flies were placed in a refrigerator (4 °C) for 3 minutes before partial dissection using hemolymph-like solution HL3 [48] and secured on a 0.5 cm diameter bed of wax on either a cover slip or on top of recessed porous platform (6 × 12 cm, porous polyethylene) capable of delivering a laminar flow of N2 gas over the entire fly. The brain was exposed by removing a small piece of cuticle along the dorsal midline of the fly head. K+ -sensitive microelectrodes were inserted through the sheath into the brain to measure [K+]o.

K+ -sensitive microelectrodes were fashioned from non-filamented glass pipettes (1 mm diameter; World Precision Instruments Inc., Sarasota, FL, USA) that were cleaned with methanol (99.9 %) and dried on a hot plate, then 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 inserted in distilled water until experimentation. Reference electrodes were made by pulling a filamented pipette (1 mm diameter; World Precision Instruments Inc., Sarasota, FL, USA) 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 an electrode holder with a chloride-coated silver wire and 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 voltage change needed to establish [K+]o using the Nernst equation. Electrode sensitivities ranged from 50 to 60 mV for a 10-fold change in K+.

**Intact animal ouabain treatment**

W^{1118} males were aged to 14-21 days. Flies were placed in an air-tight sealed vial with 10 μl of 100 mM Ouabain (Sigma-Aldrich) in DMSO (BDH) for one hour in the dark at room temperature prior to video analysis [49]. Comas were identified as periods of time longer than 5 s when individuals were lying on their sides, motionless and unresponsive to touch or vibration. Flies were monitored for 6 hrs and coma start and stop times were recorded for each individual fly, up to and including the time to permanent coma onset.

**Anoxia test**

AD was induced rapidly and reliably by passing a stream of N2 over the preparation. The majority of experiments used a protocol of repeating 2.5 minutes anoxia followed by 4 minutes recovery to measure the consequences of repetitive AD. To test the effects of increasing the duration of anoxia exposure on the shape of [K+]o, surges we slowly extended the duration from 5 to 90 s in 5 s steps.

**Immunostaining**

Immunohistochemistry was conducted according to a previously described protocol [50]. Briefly, dissected adult brains of 7-day old male flies were fixed in freshly prepared 4 % formaldehyde for 20 min at room temperature. The tissues were then washed and saturated in 5 % goat serum (Sigma) for 1 h at room temperature. Incubation with primary antibodies was performed at 4 °C for 48 h with a rabbit anti-c-myc (GenScript) at 1:50. Following the wash, tissues were incubated in secondary antibodies at 4 °C for 48 h. Secondary antibodies were DyLight 488 conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) at 1:500. The incubation tubes were wrapped with aluminum foil to keep the tissue in the dark. After three separate washes, tissues were re-mounted in 200 μl SlowFade Gold Antifade reagent (Invitrogen) and mounted on slides. Confocal images were taken with a Carl Zeiss LSM 710 NLO Laser Scanning Confocal/Multiphoton Microscope and processed with LSM software Zen 2009 (Carl Zeiss).

**Statistical analysis**

SigmaPlot 11.0 integrated with SigmaStat 3.1 was used to assess data groupings for significance. Statistical analyses used one-way and two-way repeated measures ANOVA, followed by a post-hoc Tukey multiple comparison test. For non-parametric tests a Kruskal-Wallis one way ANOVA on ranks was performed. For before and after experiments paired t-tests were performed. Significance was assessed at P<0.05 (single asterisks or daggers) however the majority of P-values are less than 0.01 (double asterisks or daggers).

**Supporting Information**

**Video S1** Footage of a adult fly exhibiting repetitive comas. The fly was exposed to volatilized ouabain (10 μl of 100 mM) for 1 hr prior to videotaping. (WMV)

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**Author Contributions**

Conceived and designed the experiments: GABA KD-S RMR. Performed the experiments: GABA JLK CX. Analyzed the data: GABA. Contributed reagents/materials/analysis tools: KD-S LS RMR. Wrote the paper: GABA RMR.

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