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2 **AMP-activated protein kinase protects against anoxia**
3 **in *Drosophila melanogaster***

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Abstract

During anoxia, proper energy maintenance is essential in order to maintain neural operation. Starvation activates AMP-activated protein kinase (AMPK), an evolutionarily conserved indicator of cellular energy status, in a cascade which modulates ATP production and consumption. We investigated the role of energetic status on anoxia tolerance in *Drosophila* and discovered that starvation or AMPK activation increases the speed of locomotor recovery from an anoxic coma. Using temporal and spatial genetic targeting we found that AMPK in the fat body contributes to starvation-induced fast locomotor recovery, whereas, under fed conditions, disrupting AMPK in oenocytes prolongs recovery. By evaluating spreading depolarization in the fly brain during anoxia we show that AMPK activation reduces the severity of ionic disruption and prolongs recovery of electrical activity. Further genetic targeting indicates that glial, but not neuronal, AMPK affects locomotor recovery. Together, these findings support a model in which AMPK is neuroprotective in *Drosophila*.

Introduction

Optimal neural function requires an uninterrupted supply of energy. Prolonged disruption of this supply can induce neuronal damage, whereas short periods can be tolerated and some organisms are more tolerant by virtue of adaptive mechanisms of metabolic suppression (Staples and Buck, 2009). *Drosophila* survive anoxic conditions, which induce severe energetic depletion, by entering a reversible coma (Lighton and Schilman, 2007; Benasayag-Meszaros et al., 2015). The coma is generated by anoxic depolarization in the brain (Armstrong et al., 2011) which is a form of spreading depolarization (SD), a loss of CNS ion gradients common to insects and mammals (Spong et al., 2016a) and associated with several important brain pathologies (Dreier and Reiffurth, 2015). During this coma, survival depends on the down-regulation of energy turnover and up-regulation of ATP-producing pathways (Hochachka et al., 1996). An enzyme known to play a role in coordinating energy allocation is the metabolic sensing protein, adenosine monophosphate-activated protein kinase (AMPK). In insect preparations AMPK activation mimics hypoxia and starvation by reducing neuronal excitability (Money et al., 2014) and exacerbating ouabain-induced SD (Rodgers-Garlick et al., 2011). Activation of AMPK contributes to mechanisms for coping with hypoxia by metabolic suppression in non-mammalian vertebrates, such as fish (Jibb and Richards, 2008; Zhu et al., 2013). AMPK has diverse roles in different mammalian tissues (Mantovani and Roy, 2011) but, in spite of much research on the role of AMPK in neural damage after oxygen limitation associated with stroke (Manwani and McCullough, 2013) there is little knowledge of its potential role in modulating induction and recovery from SD.

52 AMPK is a heterotrimeric protein consisting of a catalytic alpha (α) subunit, regulatory gamma
53 (γ), and scaffolding beta subunit (β). Allosteric activation by AMP leads to the phosphorylation
54 of the α subunit on a threonine residue (Thr-172) (Hawley et al., 1995). A rise in AMP, via the
55 adenylation kinase reaction, occurs when ATP is depleted. Since AMP:ATP varies as a square of
56 ADP:ATP, the AMPK cascade is a sensitive indicator of cellular energy change (Hardie et al.,
57 1998). Once activated, AMPK inactivates the ATP-consuming anabolic pathways: fatty acid
58 synthesis via phosphorylation of acetyl-CoA carboxylate and sterol synthesis via
59 phosphorylation of 3-hydroxy-3-methylglutaryl-CoA reductase (Hardie et al., 1999). As a result,
60 depression of acetyl-CoA carboxylate activates catabolic ATP-production via a reduction in
61 malonyl-CoA concentration. Low concentration of malonyl-CoA reduces inhibition of carnitine
62 palmitoyltransferase I (CPT-I), leading to an influx of fatty acid substrate (Guzman and
63 Blazquez, 2004). Surplus fatty acid is converted into ketone bodies to be used as a source of
64 alternative cellular energy during dietary restriction or prolonged starvation (Murray et al.,
65 2016).

66
67 Mechanisms of metabolic regulation during dietary restriction are highly conserved. In
68 mammals, dietary restriction leads to the accumulation of lipids in the liver, which are later
69 oxidized to become ketone bodies. While ketone bodies are mainly synthesized and supplied by
70 the liver, studies suggest glia exhibits hepatic-like ketogenic machinery (Blazquez et al., 1998).
71 Dietary restriction, or 'ketogenic diets,' can protect the brain from oxidative stress in mammals as
72 well as in *Drosophila* (Suzuki et al., 2001; Vigne et al., 2009; Gibson et al., 2012). In
73 *Drosophila*, the oenocytes and fat body have metabolically similar liver-like functions (Gutierrez
74 et al., 2007). Similarly, dietary restriction increases lipid accumulation in oenocytes and is
75 associated with heightened levels of ketone bodies in the brain (Johnson et al., 2010; Schulz et
76 al., 2015). While AMPK is activated in periods of dietary restriction (Slack et al., 2012), the role
77 of AMPK in these tissues during anoxic stress is still unknown.

78
79 Here we show that AMPK modulates recovery from anoxic comas in *Drosophila*. This process
80 involves the return of ion homeostasis in the brain followed by recovery of neural circuit
81 function upon return to normoxia. We found that pharmacological AMPK activation mimics the
82 effects of dietary restriction in regulating anoxic recovery and demonstrated using genetic
83 manipulations that AMPK function in glia, rather than neurons, is important for recovery of
84 locomotor circuits from SD. In addition, AMPK activity in tissues that drive metabolism
85 (oenocytes, fat body, and glia) is central to recovery from anoxia. Our results are consistent with
86 models that suggest AMPK mediates the supply of alternative neural energy and support the
87 conclusion that the AMPK cascade is neuroprotective in *Drosophila*.

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89

90 **Material & Methods**

91

92 *Drosophila culture*

93 All control lines were maintained on standard agar medium (SAM): 0.01 % molasses, 8.20 %
94 cornmeal, 3.40 % killed yeast, 0.94 % agar, 0.18 % benzoic acid, 0.66 % propionic acid and
95 86.61 % water at room temperature (25 ± 0.25 °C) and 60-70 % relative humidity. Emerging
96 flies were collected over a 48 hour period using nitrogen (N₂) anesthesia and transferred to new
97 food vials. All vials were maintained at equal densities (approx. 20 flies). Experiments were
98 performed on male flies aged 5-9 days after eclosion. Flies were given a minimum of three days
99 without N₂ exposure before experimentation to mitigate acute tolerance to anoxia.

100

101 *Fly strains*

102 *w¹¹¹⁸* flies were used as control. Tissue specific manipulation of AMPK was achieved using the
103 Gal4/UAS system. The UAS lines used to upregulate, UAS-Cherry-AMPK α (#51871), and
104 downregulate, UAS-AMPK α -RNAi (#25931), AMPK have been previously described (Swick et
105 al., 2013; Li et al., 2016).

106

107 GAL4 enhancer-trap strains with broad tissue expression included the pan-neuronal (*Elav* #8765)
108 and pan-glial (*Repo* #7415) lines. Specific glial expression lines included surface glia
109 (subperineural: *NP2276*) and neuropil glia (astrocyte: *Alrm II*, *Alrm III*, *NP1243*; ensheathing:
110 *mz0709*, *NP6520*) (see (Awasaki et al., 2008) for a detailed review). Transgene controls were
111 backcrossed to the *w¹¹¹⁸* background.

112

113 Expression patterns of mifepristone (RU486) steroid-activated Gal4 lines used are:
114 P[Switch2]GSG3448 in fat body, oenocytes, and tracheal cells; P[Switch2]GSG10751 in fat
115 body; P[Switch2]GSGB9-1 in oenocytes. All GSG lines were generated and described by
116 Nicholson *et al.* (2008), with expression patterns shown in supplemental data
117 (http://flystocks.bio.indiana.edu/Browse/gal4/gal4_switch.php).

118

119 All lines were obtained from the Bloomington Stock center (Bloomington, IN).

120

121 *Treatments*

122 (A) Starvation

123 In preliminary experiments, flies exhibited optimal survival and anoxic tolerance after 24 hrs of
124 starvation; *w¹¹¹⁸* flies were tested at 12, 24, 36 hours and during a survival assay (data not
125 shown). Under starvation conditions, flies were transferred 24 hours before testing into vials
126 containing water-soaked filter paper. The vials were rehydrated 12 hours into starvation to
127 mitigate desiccation effects.

128

129

130 (B) Pharmacology

131 Metformin (1-1 dimethylbiguanide hydrochloride; Sigma-Aldrich) was added directly into
132 heated standard agar medium, to make a 100 mM concentration, and allowed to settle for 24
133 hours before testing. AICAR (5-aminoimidazole-4-carboxamide-1-beta-D-ribose-furanoside;
134 TOCRIS) was dissolved into 1% dimethylsulfoxide (DMSO) to make a 100 mM aqueous stock.
135 Approximately 250 μ l aliquots, from the stock, were layered on the standard agar medium and
136 allowed to absorb for 24 hours before testing (Vigne et al., 2009). Controls were layered with
137 250 μ l of 1% DMSO. Flies were fed AICAR or metformin for four days prior to testing.

138

139 (C) Gene-Switch System

140 Gene-Switch flies were fed RU486 (mifepristone; Sigma-Aldrich) based on optimal time and
141 dose-dependent transcriptional activation reported (Osterwalder et al., 2001). RU486 was mixed
142 into heated standard agar medium (10 μ g/mL) and allowed to settle for 24 hours before testing.
143 Gene-Switch flies under non-starvation conditions were placed on either standard agar medium
144 or standard agar medium with RU486 for two or four days prior to testing. Gene-Switch flies
145 under starvation conditions were fed standard agar medium for at least four days and then
146 transferred 24 hours before testing into vials containing either non-nutritive agar or non-nutritive
147 agar with RU486 (10 μ g/mL).

148

149 *Locomotor Assay*

150 A high-throughput locomotor assay (Xiao and Robertson, 2015) was used to evaluate changes in
151 activity level before and after anoxia. Flies were loaded into circular chambers (1.27 cm diameter
152 x 0.3 cm height) (Fig. 1A); one fly per chamber. Video recording (15 fps) was used to capture
153 two-dimensional locomotion as the chamber restricts vertical movement (Video 1S). Fly tracking
154 software (Xiao and Robertson, 2015), was used to calculate each fly's location every 0.2 s
155 throughout the recording period. The positions (x, y) of each fly was exported to Excel
156 spreadsheets to evaluate different parameters.

157

158 The total recording included periods of baseline activity (10 min), anoxia (30 s of N₂), and post-
159 anoxic activity (60 min) (Fig. 1B). During the experiment airflow was maintained at 2 L/min to
160 avoid hypoxic conditions, and a N₂ (8 L/min) exposure was used to induce an anoxic coma. To
161 allow animals to recover from handling, the first five minutes of each baseline recording was
162 disregarded (Liu *et al.* 2007). Further, as placement in the chambers can injure the flies,
163 individuals that failed to recover 10% of regular baseline activity (250 cm / 5 min) following
164 anoxia were excluded from further analysis.

165

166 *Locomotor Activity Parameters*

167 (A) Pre-anoxia

168 Pre-anoxia parameters were used to evaluate changes in locomotor activity during the baseline
169 period. Path Length (PL) was used to measure the total distance travelled (cm) during the 5

170 minute pre-anoxia period. Time at Rest (TAR) denotes a time period, 2 seconds or longer, during
171 which the individual did not meet the defined 'activity' threshold (0.15 cm per second). This
172 'inactive' time period was recorded as a percentage of the pre-anoxia time period. The rationale
173 was that both PL and TAR could be used to evaluate the hyperactivity commonly associated with
174 starvation (Lee and Park, 2004; Johnson et al., 2010).

175

176 (B) Anoxia & Post-anoxia

177 Parameters during and after anoxia were used to characterize changes in susceptibility and
178 tolerance to anoxic conditions. The parameters which evaluated susceptibility to anoxia (30 s of
179 N₂) include Time to Succumb (TS) (Fig. 2Aii). TS was measured as the time (s) from N₂ onset to
180 the point when activity fell below the recovery threshold (< 0.30 cm per second). Recovery from
181 anoxia, denoted Time to Recovery (TR), was measured as the time taken for locomotor activity
182 to surpass a defined threshold (0.3 cm per second) at least 10 times within 60 consecutive
183 seconds (Xiao and Robertson, 2016). The earliest time to reach the criterion is set as TR (Fig.
184 2A). As normal TR occurs within 30 minutes, individuals that failed to meet the TR criterion
185 within the 60 minute post-anoxia period were excluded in order to make comparisons between
186 'recovered' individuals.

187

188 *Spreading Depolarization: DC potential shifts*

189 Flies were placed head first into a disposable pipette tip, without the use of N₂, and fixed in place
190 using a small drop of wax. Using microscissors, a small window (0.06 x 0.02 mm) was made in
191 the cuticle at the back of the head behind the ocelli (Rodriguez and Robertson, 2012). A
192 chlorided silver wire was placed into the mid-thorax to ground the preparation. Next, a
193 microelectrode pulled from a 1 mm diameter filamented glass capillary tube, backfilled with
194 1mM KAC, was inserted at an approximate 45° angle through the window into the hemolymph.
195 Further insertion of the microelectrode, from the hemolymph to the brain, was characterized by a
196 negative shift in the resting DC potential.

197

198 SD in the fly brain was recorded by monitoring DC potential shifts using a Model 2000 pH/ion
199 amplifier (World Precision Instruments Inc) using the electrophysiology software Clampex for
200 later analysis using Clampfit (Molecular Devices, version 10.3). The recording period included
201 periods of baseline activity (10 minutes), anoxia (30 seconds of N₂), and post-anoxia (20
202 minutes). Individuals that exhibited spontaneous, large amplitude DC shifts, which can result
203 from damage, during the baseline recording period were discarded. Direct airflow was
204 maintained throughout the experiment and N₂ was used to induce an anoxic coma.

205

206 *Spreading Depolarization Parameters*

207 The baseline of each recording was adjusted to zero just before the start of anoxia (10 minutes)
208 and after the major ionic disruption in order to normalize DC potential drift during the recording.
209 The time to surge (s) was measured from the time nitrogen was turned on to the point at half-max

210 amplitude of the abrupt negative shift in DC potential. The time to recover (s) was measured
211 from the time nitrogen was turned off to the point of half-max amplitude on the positive shift in
212 DC potential. The peak amplitude (mV) during the anoxia was used to quantify the magnitude of
213 spreading depolarization.

214

215 *Measurement of Mass*

216 Adult flies were placed in a freezer (~30 min) before weighing to ensure complete
217 immobilization, after which they were individually weighed to 1 μ g using a Cahn-microbalance.
218 Controls were weighed at the same time of day to account for potential water loss. Pupae were
219 weighed under similar conditions. Each pupa was dissected under a light-microscope to verify
220 sex, and genotype via eye color.

221

222 *Statistical Analyses*

223 Each locomotor activity test included all controls; for example both Gal4/+ and UAS/+ flies for
224 genetic manipulations or drug-specific controls for flies fed AMPK pharmacology. As replicate
225 data sets exhibited similar trends we compiled the data for statistical analyses.

226

227 Statistical analyses were performed using Sigma Plot 12.1 (Systat Software Inc.). Parametric
228 tests performed were a t-test and one-way ANOVA when the distribution of residuals passed
229 normality (Shapiro-Wilk). Non-parametric tests, when normality failed, included a Mann-
230 Whitney Rank Sum Test and ANOVA on Ranks (Kruskal-Wallis) with Dunn's multiple
231 comparisons. Significance levels were set at $\alpha < 0.05$ and 0.10.

232

233 **Results**

234

235 *AMPK activation mimics starvation phenotype before and after anoxia*

236 To evaluate phenotypic changes in locomotor behavior before, during, and after exposure to
237 anoxia we used a high-throughput locomotor assay and open-source software to track each fly's
238 location (Fig. 1; see also Video 1S) (Xiao and Robertson, 2015). Flies placed within the
239 restricted locomotor chamber engaged in near constant walking (Fig. 2) [Time at Rest (TAR):
240 8.7 ± 2.1 %; Path Length (PL): 215.3 ± 6.5 cm]. When deprived of food for 24 hrs the flies
241 exhibited a distinct 'starvation phenotype'. Starvation significantly decreased TAR 4.3 ± 0.36 %
242 and increased PL 235.2 ± 5.32 cm (*Mann-Whitney*, $U(31, 32) = 1184.5$, $p = 0.008$; *Mann-*
243 *Whitney*, $U(31, 32) = 827$, $p = 0.024$). When fed the AMPK activating compounds metformin
244 and AICAR, w^{1118} flies exhibited an increase in PL (Metformin: *t*-test, $t = -3.355$, $df = 30$, p
245 $= 0.001$; AICAR: *t*-test, $t = -2.088$, $df = 62$, $p = 0.021$), however only metformin decreased TAR
246 (Metformin: *Mann-Whitney*, $U(16, 16) = 62.0$, $p = 0.013$; AICAR: *Mann-Whitney*, $U(32, 32) =$
247 460.5 , $p = 0.493$). This suggests that, under normoxic conditions, pharmacological activation of
248 AMPK has a similar effect on locomotor activity as starvation.

249

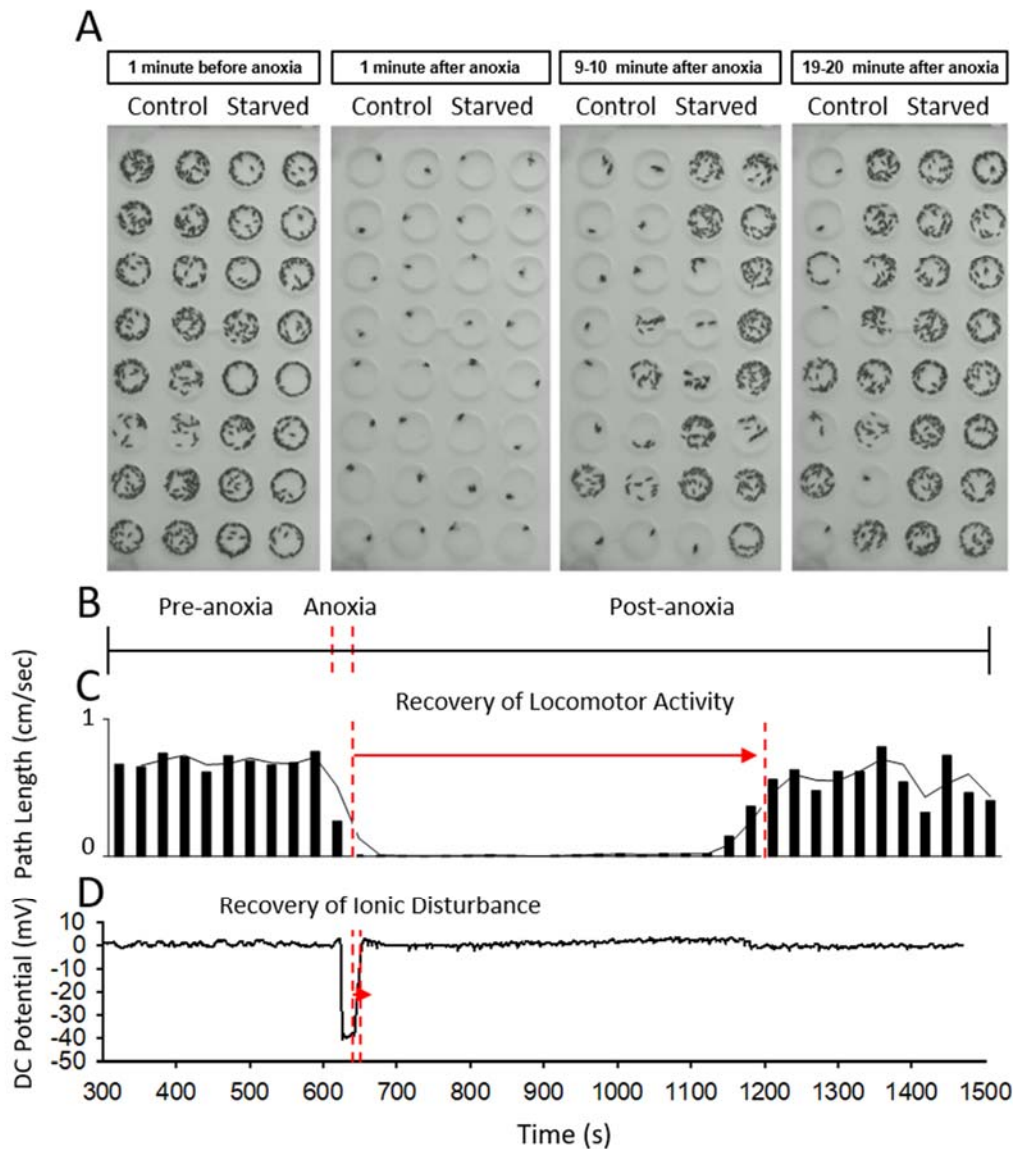


Figure 1. Locomotor recovery from anoxic coma. (A) Behavioral assay. Superimposed images (300) from 1 minute of video recording at 4 different time points before, during and after an anoxic coma. Each chamber contained a single fly. Note that starved flies recovered locomotion faster after an anoxic coma. See supplemental video, **Video 1S**. **(B)** The recording period includes baseline activity (10 min), anoxia (30 s of N₂), and post-anoxic activity (60 min). **(C)** Locomotor recovery of an individual fly indicated by average path length (cm) every 30 seconds (columns) and a moving average of path length (line). Timing of anoxia aligned with B. **(D)** Extracellular recording of DC potential (mV) from the brain. Abrupt negative DC shift indicates spreading depolarization. Note that recovery of ion gradients occurs rapidly in contrast to recovery of locomotion. Timing of anoxia aligned with B and C.

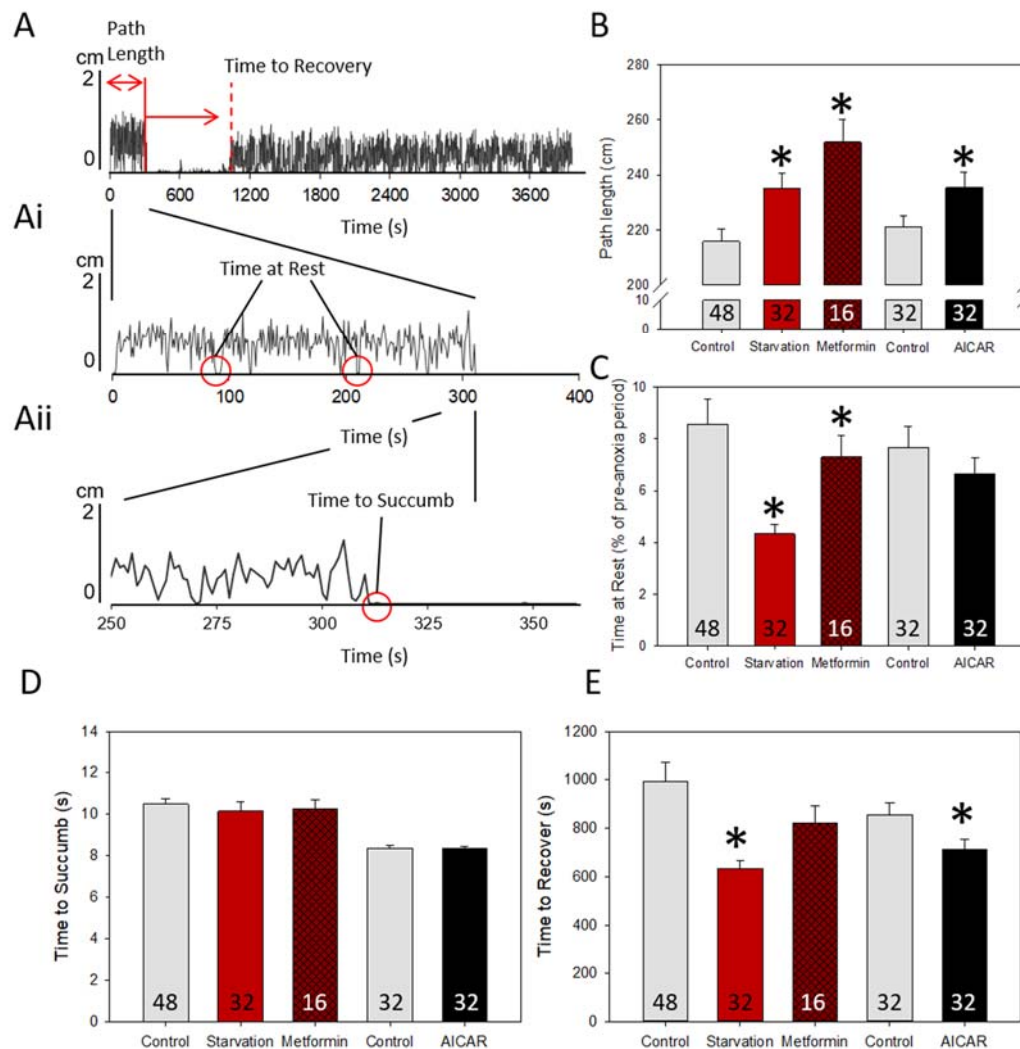


Figure 2. Starvation and AMPK activation increase locomotor activity and speed recovery from an anoxic coma. (A) **Ai** Locomotor activity parameters. Path Length (PL) is the distance (cm) traveled during the pre-anoxia period. Time at Rest (TAR) is the percentage of time during the pre-anoxia period that the individual does not meet the activity threshold (< 0.15 cm/s) for periods of at least 2 s. Time to Recovery (TR) (s) indicated by the dashed line. **Aii** During anoxia (30 s of N₂), the Time to Succumb (TS) is the time (s) from nitrogen on to a period of activity below the recovery threshold (< 0.30 cm/s). (B, C) 24 hrs of starvation significantly increased PL and decreased TAR. Pharmacological activation of AMPK by feeding 100 mM metformin or 100 mM AICAR for four days mimicked the effects of starvation. $p < 0.05$ (*) by *t*-test or Mann-Whitney Rank Sum. (D) Treatments did not affect TS. (E) Starvation and AICAR significantly reduced TR. $p < 0.05$ (*) by Mann-Whitney Rank Sum. Data are plotted as mean \pm SE. Sample sizes are indicated in the bars. Starvation and metformin controls are combined for analysis; significance comparisons were made between treatments and their respective controls.

252 When exposed to brief anoxia (30 s of N₂), w¹¹¹⁸ flies undergo stereotyped movements: rapid
253 paralysis, falling over, and inactivity. We found that the time for flies to exhibit inactivity (Time
254 to Succumb: TS) after exposure to anoxia was not significantly different after a 24 hr period of
255 starvation, or when fed Metformin or AICAR (Fig. 2D) (Starvation: *Mann-Whitney, U* (31, 32) =
256 463.0, *p* = 0.649; Metformin: *Mann-Whitney, U* (16, 16) = 113.0, *p* = 0.575; AICAR: *Mann-*
257 *Whitney, U* (32, 32) = 1072.0, *p* = 0.631). Upon return to normoxia, w¹¹¹⁸ flies starved for 24 hrs
258 or fed AICAR had a significantly shorter time to recover locomotion (Time to Recover: TR)
259 (Starvation: *Mann-Whitney, U* (25, 32) = 253.0, *p* = 0.018; AICAR: *Mann-Whitney, U* (32, 32) =
260 360.0, *p* = 0.042). While flies fed metformin showed a similar trend, treatment did not
261 significantly affect TR (*Mann-Whitney, U* (16, 16) = 89.0, *p* = 0.147). To determine if this was
262 due to neural mechanisms, we next investigated the effect of starvation and pharmacological
263 activation of AMPK on ionic disruption in the brain during anoxia.

264
265 To determine if AMPK could modify CNS tolerance to anoxia we induced a 30 s anoxic coma
266 and monitored spreading depolarization (SD) in the brain. SD refers to the loss of ion gradients
267 induced by a failure of ion homeostasis. The rise in extracellular K⁺ and other ion disturbances
268 induce a large negative shift in extracellular DC potential causing the invertebrate CNS to
269 abruptly shut down in response to acute stress (Rodriguez and Robertson, 2012; Spong et al.,
270 2016b). Anoxia induces SD in the fly brain, pre-anoxia positivity (1 s) and a large negative DC
271 shift (surge) which is sustained until N₂ is turned off (Fig. 3A), that is similar to anoxic
272 depolarization in the mammalian brain (Hansen, 1985; de Crespigny et al., 1999; Lindquist and
273 Shuttleworth, 2017). We found that the time to CNS shutdown, defined as the time to half-max
274 amplitude of the surge (s), was significantly longer when flies were starved 24 hours or fed
275 metformin (Fig. 3B) (Starvation: *t*-test, *t* = -1.981, *df* = 19, *p* = 0.03); Metformin: *t*-test, *t* = -
276 2.298, *df* = 20, *p* = 0.016). However, flies fed AICAR, which acts as a more direct activator of
277 AMPK, did not exhibit a longer time to surge (*Mann-Whitney, U* (6, 10) = 25.0, *p* = 0.625). We
278 next asked if starvation or AMPK pharmacology would affect the magnitude of SD in the fly
279 brain.

280
281 We predicted that flies with faster locomotor recovery would have smaller ionic disturbances
282 during anoxia, enabling them to re-establish ionic gradients across cell membranes faster. What
283 we found was that during anoxia, the peak amplitude was significantly smaller in flies fed
284 Metformin or AICAR, but not after a 24 hr starvation period (Fig. 3C) (Metformin: *Mann-*
285 *Whitney, U* (11, 11) = 22.0, *p* = 0.013; AICAR: *Mann-Whitney, U* (6, 10) = 14.0, *p* = 0.093;
286 Starvation: *t*-test, *t* = 0.975, *df* = 19, *p* = 0.17). However, following anoxia, flies emerging from
287 an anoxic coma, defined as the time to recover half-max amplitude (s), took significantly longer
288 when fed Metformin or AICAR (Fig. 3D) (Metformin: *Mann-Whitney, U* (11, 11) = 16.0, *p* =
289 0.004; AICAR: *Mann-Whitney, U* (6, 9) = 0.0, *p* = 0.002), while flies starved 24 hrs did not
290 exhibit a difference (*Mann-Whitney, U* (10, 11) = 51.0, *p* = 0.805).

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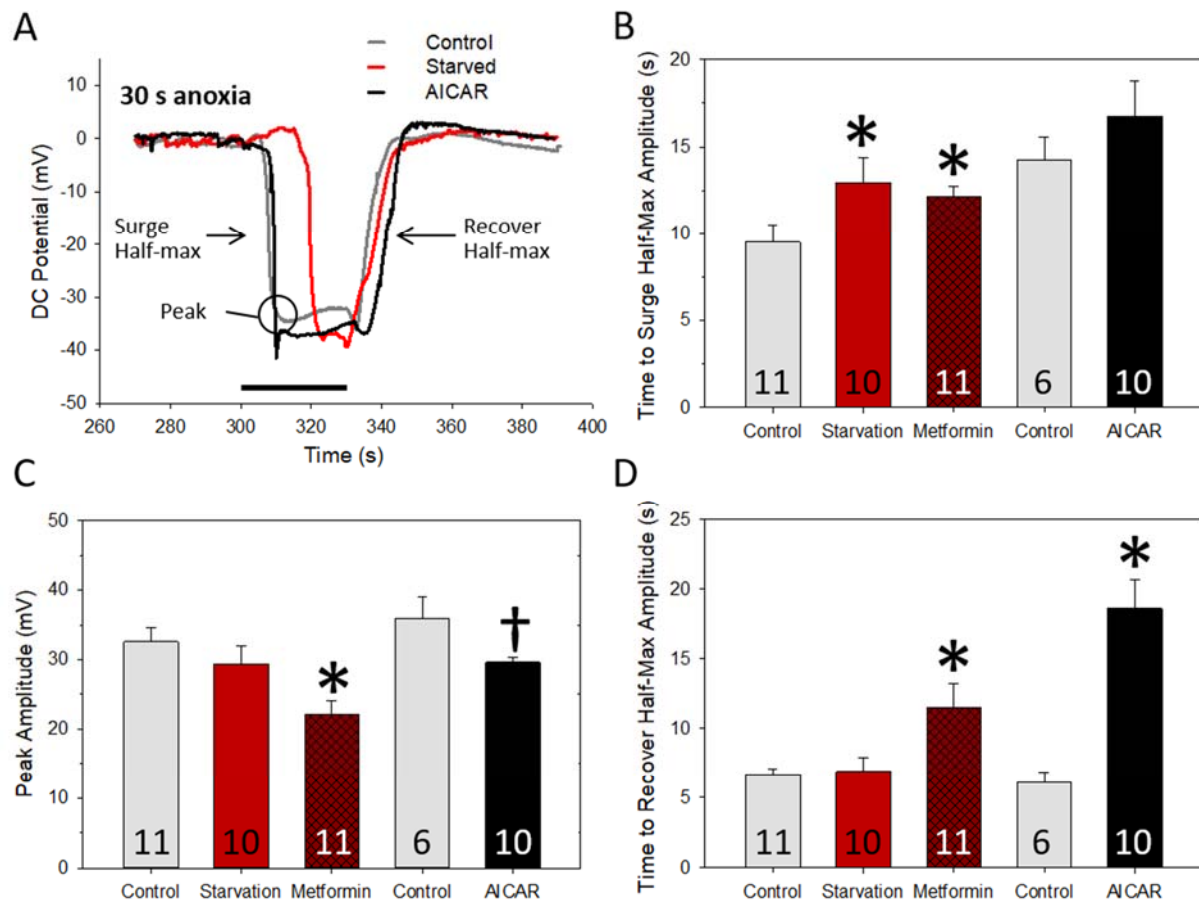


Figure 3. Starvation and AMPK activation have similar effects on anoxia-induced SD in the brain. (A) Representative extracellular recording of DC field potential showing abrupt negative shifts induced by anoxia in flies: control (grey), after 24 hrs starved (red), or fed 100 mM AICAR (black). The Peak (mV) of the DC shift was used to assess the magnitude of disruption. Time to Surge Half-Max Amplitude (s) and Time to Recover Half-Max Amplitude (s) were measured to assess the rate of failure and clearance mechanisms. (B) Time to Surge Half-Max Amplitude was longer in flies starved 24 hrs or fed 100 mM metformin. $p < 0.05$ (*) by *t*-test or Mann-Whitney Rank Sum. (C, D) Metformin or AICAR reduced peak amplitudes and increased Time to Recover Half-Max Amplitude. $p < 0.05$ (*), 0.10 (†) by *t*-test or Mann-Whitney Rank Sum. Data are plotted as mean \pm SE. Sample sizes are indicated in the bars. Starvation and metformin controls are combined for analysis; significance statistical comparisons were made between treatments and their respective controls.

293 Thus, contrary to what we expected, fast locomotor recovery did not correlate directly with
294 smaller ionic disturbance and flies with a smaller peak amplitude took longer to recover CNS ion
295 gradients after anoxia.

296

297 Although we have shown that both starvation and the AMPK activators Metformin and AICAR
298 were able to modulate SD dynamics in the brain during anoxia, it was unclear whether the
299 AMPK modulation of the speed of locomotor recovery after anoxia was mediated neurally. We
300 tested this possibility using the *Drosophila* Gal4/UAS system to up- or downregulate the AMPK
301 catalytic α subunit activity in a tissue-specific manner.

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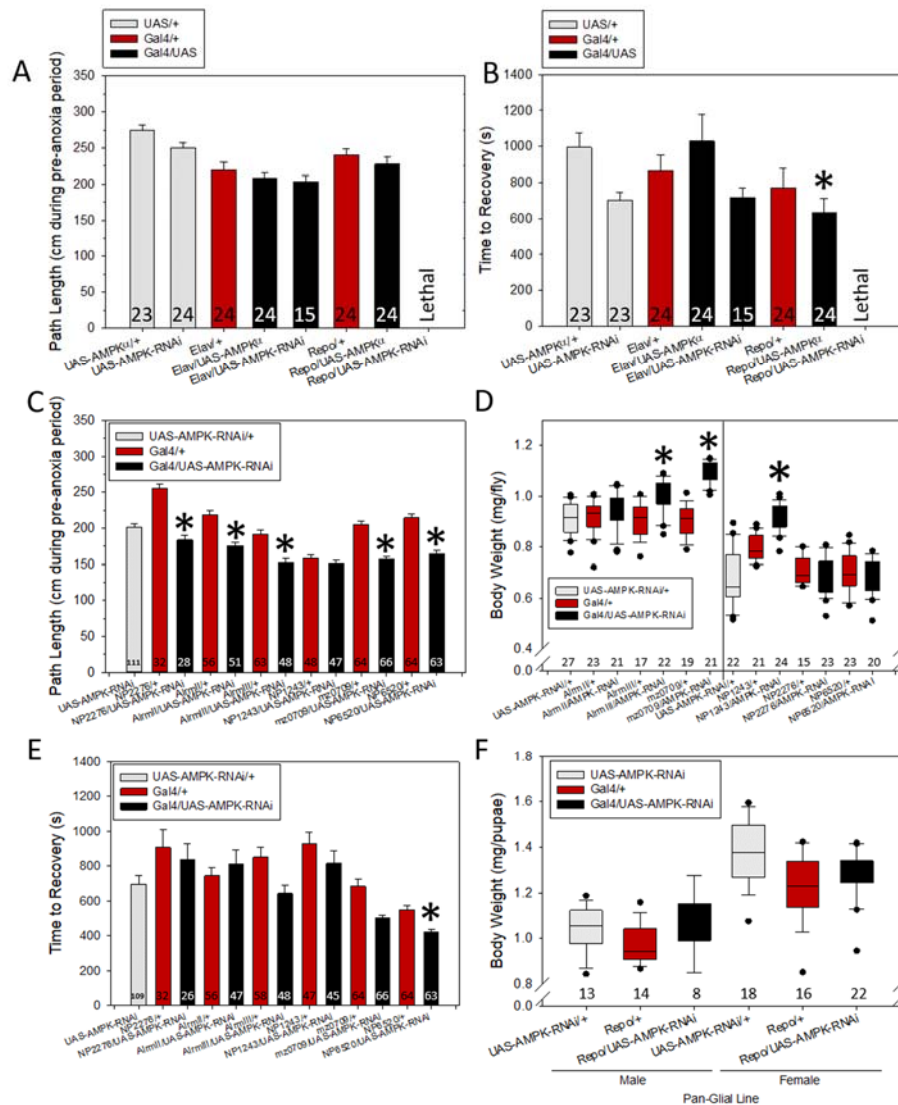
303 *AMPK α subunit required for pupation*

304 We suspected that AMPK would be required during development as *Drosophila* larvae forage in
305 a hypoxic medium (Callier et al., 2015). Complete loss of AMPK α , as seen in *ampka* mutants, is
306 lethal in late second/third instar stages (Lee et al., 2007; Swick et al., 2013). Thus, we chose to
307 restrict RNAi manipulation of AMPK α to neurons or glia. We were intrigued to find that a pan-
308 neuronal up- or down-regulation of AMPK α did not affect developmental lethality, locomotor
309 behavior, or recovery from anoxia (Fig. 4, Table 1S). In contrast, we found flies with pan-glial
310 upregulation of AMPK α had a shorter anoxic recovery time (Figure 4B) (Student-Newman-
311 Keuls Method, $Q = 3.545, 3.754, p < 0.05$), however we were unable to test the effect of pan-
312 glial AMPK α downregulation as these flies showed a male-specific lethality during pupation. As
313 *dAMPK α* mutants exhibit low triglyceride levels, small fat body cells, and decreased larval size
314 (Bland et al., 2010) we evaluated potential weight differences during pupation. We found that
315 there was no weight difference in glial AMPK-RNAi male or female flies during pupation,
316 consistent with a weight-independent lethality during pupation (Fig. 4F) (male pupae: ANOVA,
317 $p > 0.05$; female pupae: Dunn's Method, $Q = 1.926, 1.399, p > 0.05$). This suggests that
318 AMPK α may be required in glial cells to regulate pupal metamorphosis. In order to circumvent
319 this lethality we used glial subtypes to drive AMPK α -RNAi.

320

321 *Inhibition of glial AMPK α subunit induces severe metabolic defects*

322 Despite pan-glial AMPK α -RNAi lethality, males with AMPK α -RNAi in surface glia
323 (subperineural: *NP2276*) or neuropil glia (astrocyte: *Alrm II, Alrm III, NP1243*; ensheathing:
324 *mz0709, NP6520*) survived to adulthood. We found that RNAi of AMPK α in the neuropil glial
325 *Alrm III, NP1243, mz0709* increased body weight (Fig. 4D; *Alrm III*: Holm-Sidak ANOVA, $t(2)$
326 $= 4.932, 4.639, p < 0.001$; *NP1243*: Dunn's Method H (2) $= 44.033, p < 0.05$; *mz0709*: Holm-
327 Sidak ANOVA, $t(2) = 11.310, 10.852, p < 0.001$). When we evaluated locomotor behavior, we
328 also found that nearly all glial subtypes exhibited reduced locomotor activity (Fig. 4C; *Alrm II*:
329 Holm-Sidak ANOVA, $t(2) = 5.167, 3.544, p < 0.001$; *Alrm III*: $t(2) = 6.519, 4.743, p < 0.001$;
330 *mz0709*: Dunn's Method, $Q = 6.479, 6.630, p < 0.05$; *NP2276*: Holm-Sidak ANOVA, $t(2) =$
331 $6.878, 2.079, p < 0.05$; *NP6520*: $t(2) = 6.846, 2.012, p < 0.05$). While it is unclear whether
332 reduced locomotor activity in the AMPK α -RNAi glial lines was due to increased body weight, it
333 is clear that a loss of AMPK α in glia severely disrupts regular metabolic function.



335 As a higher rate of metabolism during anoxia could more rapidly deplete ATP required to
336 maintain homeostasis (Galli and Richards, 2014), we were not surprised that a low metabolic rate
337 phenotype - high body weight and low activity level - was associated with a significantly faster
338 anoxic TR in the ensheathing glial line *NP6520/UAS-AMPK α -RNAi* (Fig. 4E; Dunn's Method,
339 $Q = 5.413, 4.189, p < 0.05$). While *NP6520/UAS-AMPK α -RNAi* did exhibit a significantly
340 faster TS than its controls, a phenotype not observed in the other glial subtypes, variations in TS
341 were not associated with TR (Table 1S).

342

343 *Gene-Switch line starvation phenotype*

344 We utilized temporal and spatial downregulation of AMPK α in order to identify AMPK-
345 dependent tissues within the fly body during starvation. We chose to target AMPK α -RNAi in the
346 fat body, oenocytes, and tracheal cells which become transcriptionally inactivated when flies are
347 fed RU486 (mifepristone; Sigma-Aldrich) using Gene-Switch system lines, and scored
348 starvation-induced lethality over an 84 hour period (Osterwalder et al., 2001). Consistent with
349 research that shows pan-neuronal upregulation of AMPK shortens survival during starvation
350 (Ulgherait et al., 2014), we found that AMPK α downregulation in the GSG3448 (fat body,
351 oenocytes, and tracheal cells) or GSGB9-1 (oenocytes) lines extends survival. We also found
352 that the GSG3448 line had a lower tolerance to starvation (without RU486) (86.21% survival)
353 after 24 hours than the GSGB9-1 (100% survival) lines (Fig. 5C,D).

354

355 *AMPK α subunit affects starvation phenotype*

356 If AMPK α was critical for one of the previously described starvation phenotypes - increased PL,
357 reduced TAR, and fast TR - then its disruption using AMPK α -RNAi should abolish these effects.
358 We first characterized each line's starvation phenotype. Starvation alone (without RU486) in
359 AMPK α -RNAi Gene-Switch lines GSG3448 and GSGB9-1 caused a reduction in TAR
360 (GSG3448: *Mann-Whitney, U* (30, 31) = 349, $p = 0.095$; GSGB9-1: *t*-test, $t = 2.171, df = 24, p$
361 $= 0.020$). Following anoxic exposure, the GSGB9-1 line showed a trend towards reducing TR
362 (Table 1S), however only the combined expression line GSG3448 had a significantly reduced TR
363 after 24 hrs of starvation (GSG3448: *Mann-Whitney, U* (29, 31) = 302.5, $p = 0.030$). This
364 suggests, consistent with the results above, that these lines may have varying tolerance to
365 starvation.

366

367 When feeding RU486 to inactivate AMPK α we found that only the combined expression line
368 GSG3448 significantly affected the starvation phenotype. Starvation on agar with RU486
369 abolished the starvation-TAR phenotype; comparing starvation alone and starvation with RU486
370 (Fig. 5A) (*Mann-Whitney, U* (26, 31) = 296.5, $p = 0.088$). As well, starved GSG3448 flies no
371 longer had a fast TR when fed RU486 (Fig. 5B) (*Mann-Whitney, U* (25, 31) = 291, $p = 0.114$).
372 This suggests that during starvation the regulation of AMPK α through multiple tissues, either via
373 a cooperative or an additive effect, may be required to induce a starvation phenotype.

374

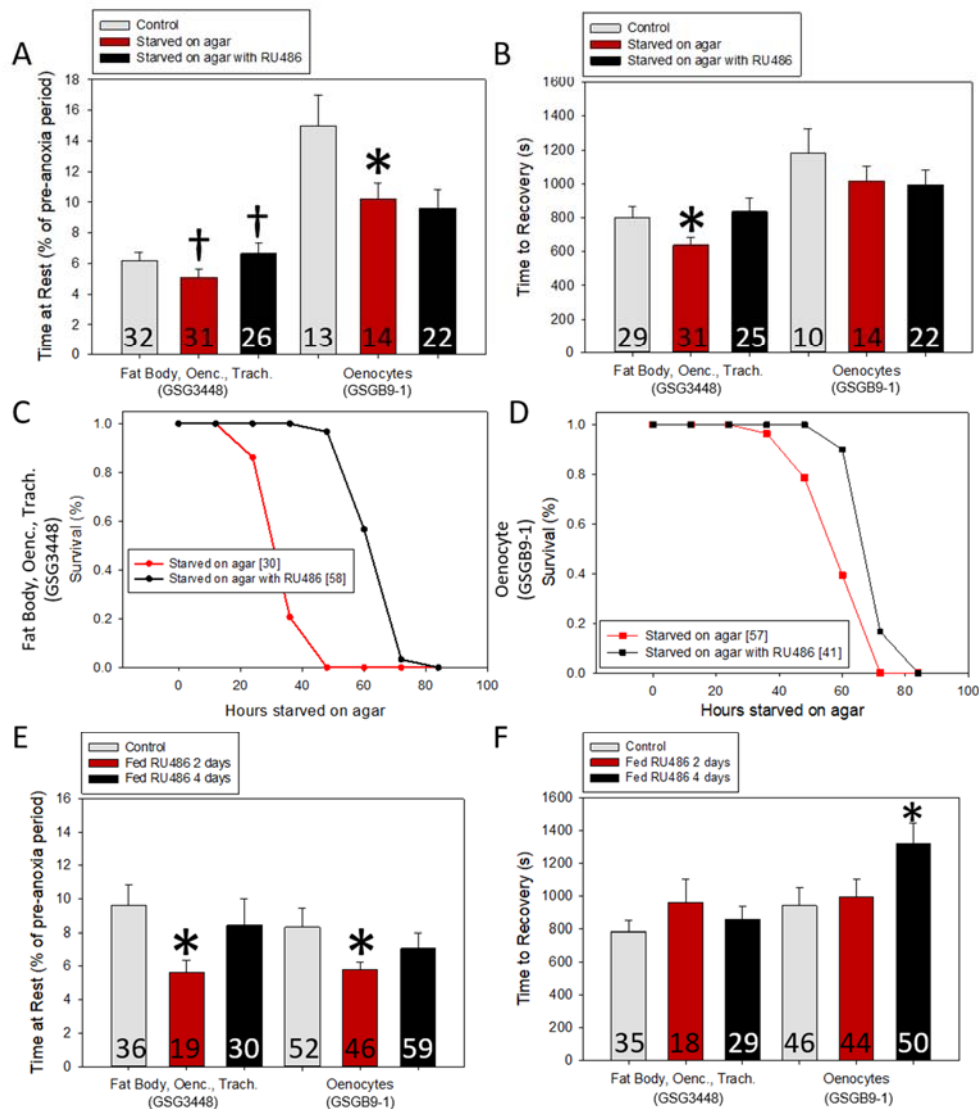


Figure 5. Effects of manipulation of AMPK α subunit expression in fat body and oenocytes with and without starvation. (A,B) Starvation (24 hr) induced hyperactivity in both the combined fat body, oenocyte, tracheal cell (GSG3448) and oenocyte (GSGB9-1) lines. Downregulation of the AMPK α subunit in the combined fat body, oenocyte, tracheal cell by adding 10 μ g/mL RU486 to the agar increased TAR and blocked the effect of starvation on anoxic TR. $p < 0.05$ (*) by Mann-Whitney Rank Sum. (C, D) Survival curves of tissue-specific lines during starvation: agar alone (red line); agar with RU486 (black line). The combined fat body, oenocyte, tracheal cell line shows improved survival on agar with RU486 compared with the oenocyte line. (E, F) Both the combined fat body, oenocyte, tracheal cell and oenocyte lines had reduced TAR after 2 days fed RU486 but did not exhibit changes in anoxic TR. After four days fed RU486 both lines exhibit regular locomotor activity, however reduced AMPK α subunit affected the oenocyte line's anoxic TR. $p < 0.05$ (*) by Mann-Whitney Rank Sum. Data are plotted as mean \pm SE. Sample sizes are indicated in bars. Significance comparisons were made as (control) vs. (starvation on agar) or (starvation on agar) vs. (starvation on agar with RU486).

376 However, as the GSG3448 line had a lower tolerance to starvation (Fig. 5C-E), we cannot rule
377 out the possibility that AMPK α is simply upregulated more after 24 hrs of starvation than the
378 single tissue line; resulting in a larger inhibitory effect of RU486.

379

380 *Short-term AMPK α subunit down-regulation affects locomotor behavior and tolerance to anoxia*

381 To address the acute effects of reduced AMPK α subunit in the oenocyte and fat body, flies were
382 fed RU486 for two and four days. We found that a two day downregulation of AMPK α in the
383 GSG3448 and GSGB9-1 lines reduced TAR, which suggests a basal level of AMPK α may be
384 active in these tissues and that the loss of AMPK α can modify locomotor behavior (Fig. 5E;
385 GSG3448: *Mann-Whitney, U* (19, 36) = 181.5, $p = 0.005$; GSGB9-1: *Mann-Whitney, U* (46, 52)
386 = 906, $p = 0.039$). However, flies fed RU486 for four days do not show this effect (GSG3448:
387 *Mann-Whitney, U* (30, 36) = 430, $p = 0.158$; GSGB9-1: *Mann-Whitney, U* (52, 59) = 1290, $p =$
388 0.151). This return to regular locomotor behavior indicates that under normoxic conditions the
389 fly may attempt to compensate for a loss of basal AMPK α within these tissues. However when
390 faced with anoxic stress, AMPK α flies fed RU486 for four days took significantly longer to
391 recover locomotor activity (TR: *Mann-Whitney, U* (46, 50) = 796, $p = 0.010$). This suggests
392 mechanisms of basal AMPK α compensation were unable to help the fly during anoxic stress;
393 which may require AMPK upregulation.

394

395 **Discussion**

396

397 We investigated how food restriction affects the response of the nervous system to anoxia in
398 adult *Drosophila*. Our main conclusions are: 1. Prior starvation speeds recovery of locomotion
399 from an anoxic coma and this is mimicked by pharmacological treatments that would activate
400 AMPK. 2. Collapse and restoration of ion gradients with anoxia/re-oxygenation are modulated
401 by starvation and AMPK. This contributes little to the timing of recovery of locomotor circuit
402 function, however, it could affect the susceptibility to SD. This remains to be determined. 3.
403 Locomotor circuit recovery after anoxia is modulated by AMPK activity in glia, particularly the
404 ensheathing glial subtype, rather than neurons. 4. AMPK in tissues that supply energy to the
405 CNS (e.g. fat body, oenocytes and glia) can modulate locomotor circuit recovery and survival
406 during periods of starvation.

407

408 We characterized a starvation phenotype of adult flies before, during, and after exposure to brief
409 anoxia and contrasted this with the pharmacological activation of AMPK. We showed that when
410 confined within a small arena, starving flies will modify their locomotor behavior to increase
411 their distance travelled by spending less time inactive. A similar hyperactivity can be seen in
412 flies fed metformin or AICAR. During anoxia, we found that starved flies are able to delay SD in
413 the brain, however this does not affect the magnitude of SD or dynamics of the recovery of ion
414 gradients. In contrast, flies fed metformin or AICAR display a smaller ionic disruption and
415 prolonged recovery following SD. As both metformin and AICAR affect adenosine - (1)

416 metformin decreases adenosine deaminase and (2) AICAR upon phosphorylation to ZMP shares
417 structural similarities with adenosine - this finding parallels work in mammals which shows that
418 SD impairs neurotransmission and that increased adenosine prolongs depression of
419 electrocorticographic activity (Ouyang et al., 2011; Lindquist and Shuttleworth, 2017).
420 Following anoxia, both pharmacological activation of AMPK and starvation improved locomotor
421 recovery rates following anoxia. As both dietary restriction and pharmacological activators of
422 AMPK have been previously shown to modulate oxidative stress in mammalian (Walsh et al.,
423 2014; Shen et al., 2017) and non-mammalian organisms (Vigne et al., 2009; LaRue and Padilla,
424 2011), here we provide a tissue-specific evaluation of AMPK in modulating anoxic tolerance.
425 We show that in the fly body, the oenocyte and fat body have independent and context-specific
426 roles in anoxic tolerance, whereas glia is the sole AMPK-dependent tissue in the brain.

427

428 *Starvation, AMPK in the liver and oenocytes*

429 Under fed conditions the fly fat body is involved in the steady transfer of lipids to the oenocytes
430 (Brasaemle, 2007). In starved flies lipids accumulate in the oenocytes (Gutierrez et al., 2007).
431 Lipid accumulation in the fly oenocytes is thought to function similar to a mammalian liver,
432 whereby lipids are mobilized to a reserve pool to 'fuel' ketogenesis, a process which provides an
433 alternative energy source to the brain during energetic stress. It has been suggested that ketone
434 bodies act as a glucose-replacing fuel, during enhanced neuronal activity, as they are
435 preferentially used for neuronal energy (Izumi et al., 1997; Blazquez et al., 1999). It has been
436 previously reported that ketone bodies protect the brain from hypoxia and ischemic injury (Go et
437 al., 1988; Auestad et al., 1991). Here we provide data that show, consistent with a pathway from
438 lipid accumulation to neuroprotective ketogenesis, that starvation modifies anoxic tolerance.

439

440 As the 'metabolic load' of the mammalian liver is split between the oenocyte and fat body in
441 flies, we sought to determine whether AMPK in these tissues affects starvation-induced anoxic
442 tolerance. Previous work investigating lipogenesis in *Drosophila* has shown that AMPK acts as a
443 master switch for lipid regulation: AMPK upregulation, through the feeding of metformin,
444 decreases total lipid content in the fly; and AMPK downregulation via the expression of a
445 dominant negative alpha subunit variant, increases lipid content in the fly oenocytes (Johnson et
446 al., 2010; Slack et al., 2012). Following this logic, decreasing AMPK during starvation should
447 increase available lipid content for ketogenenic oxidation, and further increase anoxic tolerance.
448 However, we found that when we disrupted AMPK α in the combined fat body, oenocytes, and
449 tracheal cells line, but not the oenocyte line, the beneficial aspects of starvation are lost. This
450 suggests that lipid accumulation is likely not the sole factor which allows starved individuals to
451 tolerate anoxic bouts, but rather AMPK-dependent mechanisms in the fat body. AMPK has been
452 shown to phosphorylate acetyl-CoA carboxylase (ACC1, ACC2) which turns off fatty acid
453 synthesis and turns on fatty acid oxidation in an effort to conserve energy while producing ATP
454 (Hardie, 2007). We therefore suggest that under global energetic stress, as induced by prolonged

455 starvation, the AMPK-dependent shutdown of energetically expensive pathways outweighs the
456 production of alternative catabolic pathways.
457 In unstressed, fed conditions the disruption of AMPK α in oenocytes had a time-dependent effect
458 on tolerance to anoxic conditions. We report that downregulation of AMPK α for two days in
459 either the combined fat body, oenocyte, and tracheal cell or oenocyte lines increased
460 hyperactivity, and note that this trend was diminished by day four. Our observation is consistent
461 with multiple observations that AMPK alters locomotor behavior (Lee and Park, 2004; Johnson
462 et al., 2010; Ahmadi and Roy, 2016; Moller et al., 2016). We suspect that by day four regular
463 behavior returns to normal due to the upregulation of other AMPK subunits, as has been seen in
464 mice β 2 KO mice (Steinberg et al., 2010), or through an independent compensatory pathway.
465 Despite a return to regular locomotor behavior, we found that flies with reduced AMPK α in the
466 oenocytes alone take a longer time to recover from anoxia; suggesting even in fed conditions a
467 prolonged reduction of AMPK α in the oenocytes affects susceptibility to anoxic stresses.

468 469 *Neuronal AMPK in anoxic tolerance*

470 While AMPK-driven ketogenesis is primarily thought to be derived from hepatic tissue, several
471 studies have explored ketogenic machinery in neuronal tissue. In mammals AMPK is primarily
472 expressed in neurons with some expression in astrocyte glia (Turnley et al., 1999; Culmsee et al.,
473 2001). In particular, astrocyte glia has been shown to exhibit similar ketogenic machinery as
474 hepatocytes (reviewed in (Guzman and Blazquez, 2004)): a preference for fatty acids over
475 glucose as a primary metabolic fuel during ketogenesis (McGarry and Foster, 1980; Blazquez et
476 al., 1998); carnitine palmitoyltransferase I (CPT-I) is the metabolic pace-setting step of
477 ketogenesis (Drynan et al., 1996; Blazquez et al., 1998); similar ketogenic inhibitors, malonyl-
478 CoA for CPT-I and acetyl-CoA carboxylase for malonyl-CoA, are observed in both astrocytes
479 and hepatocytes (McGarry and Brown, 1997; Blazquez et al., 1998). However, as a primary
480 signaling molecule, AMPK has also been shown to protect against neurotoxicity (Eom et al.,
481 2016), increase neuronal survival following glucose deprivation or chemical hypoxia (Culmsee
482 et al., 2001), mediate macrophage phagocytosis (Quan et al., 2015), and increase autophagy post-
483 stroke to decrease infarct volume (Shen et al., 2017), amongst other findings. While we don't
484 address the mechanisms which modulate anoxic tolerance in *Drosophila*, we do report,
485 consistent with these findings, that upregulating AMPK α in glia affects locomotor recovery
486 following anoxia. Further, to the best of our knowledge, our finding that AMPK α neural up- or
487 down-regulation in *Drosophila* does not affect post-anoxia recovery has not been previously
488 reported. Our data in combination with work in the fly showing glial but not neural Hsp70
489 mitigates loss of ion homeostasis during repetitive anoxic stress (Armstrong et al., 2011), suggest
490 that in contrast to mammalian models, in *Drosophila*, glia is an effective target to manipulate
491 anoxic tolerance in the fly brain.

492

493 In addition to evaluating neuronal AMPK up-regulation in anoxia tolerance in *Drosophila*, we
494 suspected RNAi knockdown would prolong locomotor recovery times. First, we found that

495 AMPK-RNAi was shown to be lethal in pan-glial but not pan-neural tissue during pupation. As
496 AMPK inhibition was previously shown to block glycolysis in cultured mammalian astrocytes
497 but not neurons (Funes et al., 2014), we were not surprised to find that others have shown
498 dsRNA directed against components of the glycolytic pathway - trehalose, aldolase, and pyruvate
499 - resulted in late larval and early pupal lethality in pan-glial but not pan-neural tissue in
500 *Drosophila* (Volkenhoff et al., 2015). To avoid AMPK-RNAi pan-glial lethality, we targeted
501 specific glial subtypes. Contrary to what we suspected, flies seemed to recover faster from
502 anoxia. However, nearly all glial-specific knockdown lines had lower locomotory activity and
503 higher body weight. This finding was again consistent with Volkenhoff *et al.*'s 2015 work, which
504 showed that a knockdown of components in the glycolytic pathway that did not cause lethality
505 showed severe locomotor defects when expressed in glia. Together these findings highlight the
506 importance of the AMPK pathway in glia but not neurons for pupal development and locomotor
507 behavior. Unfortunately, due to the likely changes in metabolism in these lines there is little we
508 can say about AMPK disruption during anoxia without first uncoupling the existing phenotype
509 changes. This work raises many questions as to not only what role glial AMPK plays in pupal
510 development, but also how AMPK functions in these glial subtypes to regulate regular metabolic
511 rate.

512

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514

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518

519 **Competing Interests**

520

521 The authors declare no conflicts of interest.

522

523 **References**

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694 **Supplementary Information**

695

696 **Table 1S. Summary of locomotor parameters for all tests.** Due to the exclusion criteria
 697 following anoxia, the sample size for TR is smaller in certain cases. Statistical comparisons were
 698 made between their respective controls (as outlined in previous figures). $p < 0.10$ (‡), $p < 0.05$
 699 (*), $p < 0.01$ (**), $p < 0.001$ (***)). Details of statistical tests performed are included in the
 700 Results section.
 701

	Treatment	n	Pre-anoxia Period		Anoxia	Post-anoxia Period	
			Path Length (cm)	Time at Rest (%)	Time to Succumb (s)	Time to Recovery (s)	n (TR)
Starvation	Starvation (control)	32	215.3 ± 6.5	8.7 ± 2.1	10.5 ± 0.4	874.9 ± 82.0	25
	Starvation	32	235.2 ± 5.3 *	4.3 ± 0.4 *	10.2 ± 0.4	631.6 ± 36.2 *	32
Pharmacology	Metformin (control)	16	216.8 ± 6.5	12.0 ± 1.9	10.5 ± 0.4	1178.8 ± 151.3	16
	Metformin	16	252.0 ± 8.2 **	7.3 ± 0.8 *	10.3 ± 0.5	823.2 ± 69.8 †	16
	AICAR (control)	32	221.3 ± 4.0	7.7 ± 0.8	8.4 ± 0.1	855.7 ± 49.2	32
	AICAR	32	235.5 ± 5.5 *	6.7 ± 0.6	8.3 ± 0.1	710.2 ± 43.3 *	32

Driver	Genotype	n	Path Length (cm)	Time at Rest (%)	Time to Succumb (s)	Time to Recovery (s)	n (TR)
Pan-neural	8765/+	24	219.4 ± 10.6	10.1 ± 2.4	8.0 ± 0.1	864.8 ± 88.2	24
	8765/ UAS-Cherry-AMPKα	24	208.8 ± 7.2	6.5 ± 0.7	8.3 ± 0.2	1030.7 ± 146.0	24
	8765/UAS-AMPK-RNAi	15	203.3 ± 8.8	6.1 ± 1.5	8.3 ± 0.3	717.2 ± 55.9	15
Pan-glial	7415/+	24	240.5 ± 8.0	7.90 ± 1.2	8.1 ± 0.1	770.1 ± 108.7	24
	7415/UAS-Cherry-AMPKα	24	227.7 ± 9.4	8.5 ± 0.9	7.8 ± 0.09	634.3 ± 77.6 *	24
Upstream Sequence	UAS-Cherry-AMPKα/+	24	250.1 ± 7.2	4.6 ± 0.5	8.8 ± 0.4	703.1 ± 43.7	24
	UAS-AMPKα-RNAi/+	23	274.3 ± 7.8	5.9 ± 1.3	8.2 ± 0.1	994.9 ± 78.2	23

Glial Class Driver	Genotype	n	Path Length (cm)	Time at Rest (%)	Time to Succumb (s)	Time to Recovery (s)	n (TR)
Subperineural	NP2276/+	32	255.7 ± 12.7	5.4 ± 0.5	9.1 ± 0.1	906.5 ± 104.1	32
	NP2276/UAS-AMPKα-RNAi	28	183.6 ± 13.5 *	8.1 ± 2.0	8.6 ± 0.5	837.1 ± 88.6	26
Astrocyte	NP1243/+	48	158.2 ± 9.2	13.3 ± 1.3	8.5 ± 0.4	929.3 ± 62.1	47
	NP1243/UAS-AMPKα-RNAi	47	151.3 ± 9.8	8.5 ± 0.6	8.3 ± 0.3	813.5 ± 72.6	45
Ensheathing	NP6520/+	64	214.3 ± 10.7	6.1 ± 0.5	8.5 ± 0.2	548.8 ± 25.6	64
	NP6520/UAS-AMPKα-RNAi	63	164.7 ± 8.6 ***	6.2 ± 0.6	7.6 ± 0.3 *	419.0 ± 19.7 *	63
	MZ0709/+	64	204.7 ± 10.4	9.3 ± 0.8	8.9 ± 0.2	683.0 ± 40.7	64
	MZ0709/UAS-AMPKα-RNAi	66	157.6 ± 7.3 *	5.8 ± 0.5	8.2 ± 0.3	503.2 ± 13.2	66
	Alrm II/+	56	218.6 ± 12.8	4.0 ± 0.4	9.2 ± 0.2	744.0 ± 47.3	56
	Alrm II/UAS-AMPKα-RNAi	51	175.7 ± 10.2 ***	5.1 ± 0.6	8.0 ± 0.3	808.2 ± 83.8	47
	Alrm III/+	63	191.9 ± 10.9	7.0 ± 0.9	9.0 ± 0.1	849.6 ± 57.3	58
Alrm III/UAS-AMPKα-RNAi	48	152.2 ± 13.2 ***	11.5 ± 1.7 *	8.2 ± 0.3	641.8 ± 49.5	48	
Upstream Sequence	UAS-AMPKα-RNAi/+	111	201.4 ± 8.1	5.5 ± 0.5	8.6 ± 0.2	695.3 ± 49.7	109

GeneSwitch Lines	Genotype (Driver/UAS-AMPKα-RNAi) & Treatment	n	Path Length (cm)	Time at Rest (%)	Time to Succumb (s)	Time to Recovery (s)	n (TR)
Fat body, oenocytes, and tracheal cells	GSG3448 (control)	32	174.2 ± 4.4	6.2 ± 0.5	7.5 ± 0.2	796.9 ± 71.4	29
	GSG3448 (starved on agar)	31	173.8 ± 4.1	5.1 ± 0.6 †	7.7 ± 0.2	639.2 ± 43.6 *	31
	GSG3448 (starved on agar with RU486)	26	175.5 ± 5.8	6.6 ± 0.7 †	7.8 ± 0.2	836.3 ± 80.2 †	25
Oenocytes	GSGB9-1 (control)	13	121.5 ± 8.9	15.0 ± 2.0	6.3 ± 0.8	1180.1 ± 144.6	10
	GSGB9-1 (starved on agar)	14	118.2 ± 5.8 *	10.2 ± 1.1 *	6.6 ± 0.9	1017.9 ± 85.1	14
	GSGB9-1 (starved on agar with RU486)	22	131.0 ± 5.2 †	9.6 ± 1.2	7.6 ± 0.4	994.9 ± 88.9	22
Fat body	GSG10751 (control)	14	144.7 ± 7.4	11.2 ± 3.5	8.6 ± 0.2	1153.5 ± 158.8	13
	GSG10751 (starved on agar)	20	124.3 ± 5.2	14.3 ± 2.0 *	7.5 ± 0.6	933.0 ± 82.4	20
	GSG10751 (starved on agar with RU486)	16	109.3 ± 6.4 †	20.2 ± 3.7	6.5 ± 0.5 *	1102.5 ± 155.3	15

GeneSwitch Lines	Genotype (Driver/UAS-AMPKα-RNAi) & Treatment	n	Path Length (cm)	Time at Rest (%)	Time to Succumb (s)	Time to Recovery (s)	n (TR)
Fat body, oenocytes, and tracheal cells	GSG3448 (control)	36	160.6 ± 3.7	8.3 ± 0.7	7.2 ± 0.3	784.5 ± 68.7	35
	GSG3448 (fed RU486 2 days)	19	165.2 ± 5.7	5.6 ± 0.7	8.0 ± 0.4	960.4 ± 139.6	18
	GSG3448 (fed RU486 4 days)	30	153.6 ± 5.3 †	8.4 ± 1.6	6.7 ± 0.3 †	857.0 ± 78.8	29
Oenocytes	GSGB9-1 (control)	52	159.6 ± 3.8	8.3 ± 1.1	7.4 ± 0.2	942.0 ± 106.2	46
	GSGB9-1 (fed RU486 2 days)	46	160.7 ± 3.5	5.8 ± 0.5 *	7.5 ± 0.1	993.9 ± 106.0	44
	GSGB9-1 (fed RU486 4 days)	59	161.7 ± 4.3	7.1 ± 0.9	7.4 ± 0.2	1319.7 ± 124.7 †	50
Fat body	GSG10751 (control)	38	165.6 ± 4.1	6.6 ± 0.5	7.8 ± 0.2	736.2 ± 64.3	36
	GSG10751 (fed RU486 2 days)	30	183.2 ± 6.9 †	5.4 ± 0.5	7.8 ± 0.4	605.1 ± 57.5 †	30
	GSG10751 (fed RU486 4 days)	40	171.8 ± 4.3 †	6.4 ± 0.6 †	8.0 ± 0.1	783.2 ± 63.5	39

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704 **Video 1S. Locomotor activity before, during and after anoxia in fed and starved adult flies.**

705 The video shows four columns of eight locomotor assay chambers (1.27 cm diameter) each
706 containing a single fly. The first two columns contain flies that have been fed (Control); the
707 second two columns contain flies that have been maintained without food, but with water, for 24
708 hours (Starved). The first 30 s of the video shows 5 minutes of activity with playback at 10x the
709 acquisition rate. The next 30 s is played at normal speed for the duration of the nitrogen
710 application. Note the animals entering a coma after 10 – 15 s of anoxia. The final 3 mins shows
711 30 minutes of playback at 10x speed to show post-anoxic recovery of locomotor function. Note
712 that starved flies are more active during the baseline activity and recover from anoxia faster than
713 control flies.

714