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Report

Overexpression of a *Drosophila* Homolog of Apolipoprotein D Leads to Increased Stress Resistance and Extended Lifespan

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Summary

Increased Apolipoprotein D (ApoD) expression has been reported in various neurological disorders, including Alzheimer's disease, schizophrenia, and stroke, and in the aging brain [1]. However, whether ApoD is toxic or a defense is unknown. In a screen to identify genes that protect *Drosophila* against acute oxidative stress, we isolated a fly homolog of ApoD, *Glial Lazarillo* (*GLaz*). In independent transgenic lines, overexpression of *GLaz* resulted in increased resistance to hyperoxia (100% O_2) as well as a 29% extension of lifespan under normoxia. These flies also displayed marked improvements in climbing and walking ability after sublethal exposure to hyperoxia. Overexpression of *Glaz* also increased resistance to starvation without altering lipid or protein content. To determine whether *GLaz* might be important in protection against reperfusion injury, we subjected the flies to hypoxia, followed by recovery under normoxia. Overexpression of *GLaz* was protective against behavioral deficits caused in normal flies by this ischemia/reperfusion paradigm. This and the accompanying paper by Sanchez et al. [2] (in this issue of *Current Biology*) are the first to manipulate the levels of an ApoD homolog in a model organism. Our data suggest that human ApoD may play a protective role and thus may constitute a therapeutic target to counteract certain neurological diseases.

Results

Overexpression of *GLaz* Confers Resistance to Hyperoxia, as Well as Lifespan Extension in Normoxia

Oxidative stress has been widely implicated as an underlying mechanism in the pathology of neurodegenerative disorders, including Alzheimer's disease (AD) and schizophrenia [3, 4], as well as in the injury resulting from stroke [5]. In addition, an overwhelming body of evidence supports the idea that reactive oxygen species (ROS) are a major cause of aging [6]. As the formation of ROS is a function of ambient oxygen concentration [7], exposure to hyperoxia (100% O_2) offers an attractive model for physiological studies of oxidative stress.

We therefore performed a screen to identify genes that confer increased resistance to hyperoxia. This and all the subsequent experiments in this report were performed with male flies only. We drove high-level expression of various genes by crossing enhancer promoter (EP) element lines [8] having known insertion sites with a driver line providing a ubiquitous GAL4 source, namely *daughterless* (*da*-GAL4). One of the lines, *EP(2)2383*, carrying an insertion on the second chromosome, displayed a striking GAL4-dependent phenotype and was chosen for further study. Flies carrying both *EP(2)2383* and *da-GAL4* are much more resistant to hyperoxia (Figure 1A). The insertion in *EP(2)2383* is 1.5 kb upstream of *Glial Lazarillo* (*GLaz*). RT-PCR confirmed that the *EP(2)2383* insertion causes a GAL4-dependent upregulation of the *GLaz* transcript (see Figure S1 in the Supplemental Data available with this article online). A microarray showed a 7-fold upregulation of the *GLaz* transcript, while nearby genes (*Nrkl, TppII, SptI*, and *CG17724*) in the genomic region were not affected (data not shown).

Glial Lazarillo (*Glaz*) is a *Drosophila* homolog of human Apolipoprotein D (ApoD), with 40% identity and 80% similarity to the human protein. Both are predicted to be very similar to the ancestral proteins that gave rise to the lipocalin family [9–11]. These are cup-shaped soluble molecules, able to carry hydrophobic ligands in a 1:1 molar ratio. The physiological ligand for ApoD is undetermined, although, based on binding studies, arachidonic acid and steroids are the best candidates [12]. In Alzheimer's disease, both mRNA and protein levels of ApoD are dramatically increased (350% in the cerebrospinal fluid), and the protein is recruited to the amyloid plaques [13–15]. After crush injury of the rat sciatic nerve, regeneration is accompanied by a 500-fold upregulation of ApoD at the site of the lesion for several weeks after the injury [16, 17]. Neuronal degeneration induced by kainic acid triggers an upregulation of ApoD in the affected area [18]. An increase in ApoD expression has been detected in a murine model of Niemann-Pick disease type C (NPC), an inherited lysosomal cholesterol disorder [19]. Elevated ApoD expression has been reported in schizophrenia and bipolar disorder [20]. ApoD levels have also been reported to increase with aging in both humans and mice [15, 21].

In general, there is a strong correlation between resistance to oxidative stress and increased longevity [6]. Therefore, we measured the lifespan under normoxia of flies carrying *EP(2)2383* and *da*-GAL4. Overexpression of *GLaz* led to an increase in mean lifespan at 29ºC of 18% ($p < 0.001$, Figure 1B).

Tissue-Specific Overexpression of *GLaz*

To study the overexpression further and confirm whether overexpressing *GLaz* was sufficient to cause the lifespan extension, we generated independent transgenic lines carrying the *Glaz* cDNA under GAL4 regulation. The complete *Glaz* cDNA was cloned in the pUAST vector, thereby placing the insert under the control of UAS

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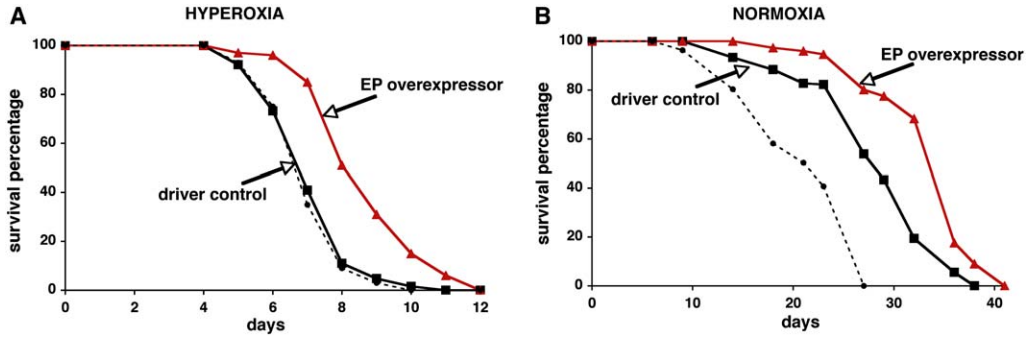

Figure 1. Increase in Hyperoxia Resistance and Extension of Lifespan by Overexpression of *GLaz*

(A) Survival under hyperoxia (100% O_2). *da*-GAL4 (ubiquitous GAL4 driver) is used to activate the UAS sequences in *EP(2)2383*. Each graph represents a trial with at least 100 flies, typical of several repeated experiments. The EP overexpressor (w^{1118}; *EP(2)2383/+; da-GAL4/+,* red curve) lives 20% longer ($p < 0.001$) than the driver control (w^{1118}; *da-GAL4/+,* black curve). The dotted line represents the EP alone (w^{1118}; *EP2383/+*).
(B) At 29ºC, under normoxia, the EP overexpressor lives 18% longer than driver control.

sequences within the boundaries of a P element. Two independent transformants were generated (*UAS-GLaz1* and *UAS-GLaz2*), both on the second chromosome. RT-PCR confirmed that both lines displayed GAL4-dependent upregulation of the *Glaz* transcript (Figure S2). Based on its relatively higher level of inducibilty, *UAS-GLaz2* was selected for further studies.

To address whether specific tissues are responsible for the lifespan extension, *UAS-GLaz2* and *EP(2)2383* were crossed with GAL4 drivers having preferential patterns of expression in various tissues. Since GAL4 generally yields stronger effects at 29ºC [22], we chose to perform these experiments at that temperature. The control used was w^{1118}, the host strain in which both UAS-*Glaz* contructs were generated. The results are outlined in Table 1. As a rule, the driver controls exhibited longer lifespans than the EP or transgenic controls, presumably due to hybrid vigor. Therefore, we systematically used the driver control as the reference lifespan for the percentage increase. Best results were obtained with relatively weak nervous system expression, combined with some expression in thoracic or abdominal muscles (*PO163*-GAL4, $GAL4^{109(2)80}$). Drivers with expression primarily in the fat or in the muscles (*DJ634*, *24B*) caused extension of lifespan, as compared to the control crosses to w^{1118}. *GMR-GAL4* also induced a robust extension of lifespan. Although the latter is commonly described as an eye-specific driver, we note that it also drives strong expression of a reporter in the salivary gland. When either the EP line or the cDNA transgene was driven by strong nervous system drivers *D42-GAL4* or *Elav-GAL4,* there was no beneficial effect. No lifespan extension was observed with *Actin-GAL4* or *Hsp70-GAL4* when driving the cDNA transgene, although *Actin-GAL4* did give a notable extension of lifespan when driving the EP line.

One of the drivers, $GAL4^{109(2)80}$, resulted in a particularly strong extension of lifespan with both the EP and the cDNA line. $GAL4^{109(2)80}$ is expressed especially in cells in the thoracic ganglion and the brain and in the vertical flight muscles of the thorax (Figure 2A). While this pattern is maintained throughout adulthood (L. Seroude, personal communication), during development it is expressed in only a small subset of sensory neurons [23]. Flies carrying $GAL4^{109(2)80}$ and *EP(2)2383* displayed a 29% increase in mean lifespan compared with the driver crossed to w^{1118} (Figure 2B), while driving *UAS-GLaz2* resulted in a 23% increase (Figure 2C).

Flies carrying both $GAL4^{109(2)80}$ and *UAS-GLaz2* displayed a 12% increase in survival under hyperoxia

Table 1. Lifespan Analysis of Tissue-Specific GAL4 Driver Lines

Driver		Control (w^{1118})	*GLaz* Overexpressor (*EP(2)2383*)		*GLaz* Overexpressor (*UAS-GLaz2*)	
		Mean ± SEM	Mean ± SEM	% Diff	Mean ± SEM	% Diff
CNS/muscle	*PO163*	25.6 ± 0.6	30.4 ± 0.6	18%, $p < 0.001$	27.8 ± 0.8	8%, $p < 0.001$
	$GAL4^{109(2)80}$	25.3 ± 0.6	32.5 ± 0.5	29%, $p < 0.001$	31.0 ± 0.5	23%, $p < 0.001$
Fat/muscle	*DJ634*	22.8 ± 0.6	26.6 ± 0.6	17%, $p < 0.001$	26.5 ± 0.6	16%, $p < 0.001$
	24B	23.2 ± 0.5	25.5 ± 0.6	10%, $p < 0.001$	29.7 ± 0.7	28%, $p < 0.001$
Eye/salivary gland	*GMR*	25.4 ± 0.6	31.0 ± 0.3	22%, $p < 0.001$	31.5 ± 0.4	23%, $p < 0.001$
Ubiquitous	*Actin*	25.6 ± 0.8	30.9 ± 0.5	21%, $p < 0.001$	26.6 ± 0.5	4%, n.s.
	Hsp70	30.3 ± 0.5	30.3 ± 0.5	0%, n.s.	29.9 ± 0.5	−1%, n.s.
Neuronal	*Elav*	31.0 ± 0.6	31.4 ± 0.7	1%, n.s.	29.3 ± 0.6	−5%, n.s.
	D42	20.2 ± 0.5	19.1 ± 0.5	−5%, n.s.	21.1 ± 0.8	4%, n.s.

The different drivers are grouped by expression patterns. The first column shows the mean lifespan ±SEM of each driver alone (crossed to w^{1118}) as controls. The second column shows the mean lifespan ±SEM of the various drivers crossed to *EP(2)2383*, and the percentage difference with the driver control (n.s. = not significant). The third column displays the same information for each driver crossed to the transgenic cDNA insertion line *UAS-GLaz*[2]. Drivers that did not have a strong expression during development all gave good extensions of lifespan. The same drivers that extended lifespan of the EP line also increased lifespan of the cDNA transgenic line, with the exception of Actin-GAL4.

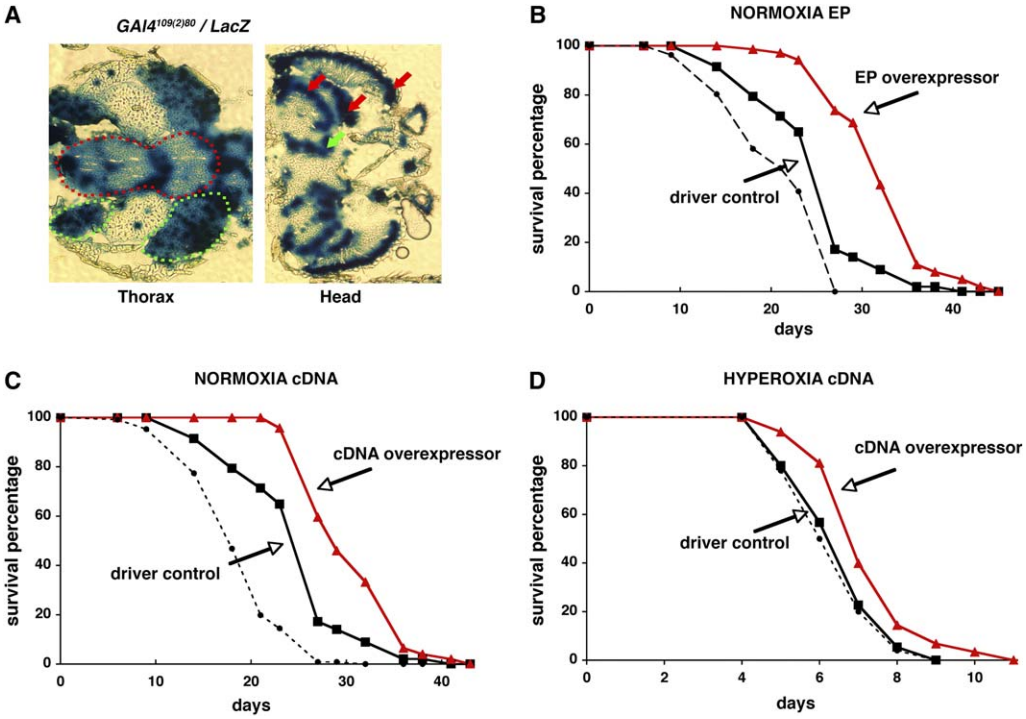

Figure 2. Protection against Hyperoxia and Extension of Lifespan by Overexpression of GLaz with *GAL4*$^{109(2)80}$ as the Driver

(A) X-GAL staining of a horizontal cryosection showing the adult expression pattern of the driver, with nuclear-LacZ under UAS control as a reporter. In the thorax, *GAL4*$^{109(2)80}$ is expressed in the two vertical flight muscles (green dotted line) and in cells of the ventral ganglion (red dotted line). In the head, the driver is expressed predominantly in the retina and optic lobes (red arrows) and central brain areas (green arrow).

(B) EP line lifespan extension under normoxia, at 29ºC. *GLaz* EP overexpressors (*w*^{1118}; *GAL4*$^{109(2)80}$/*EP(2)2383*, red curve) live 23% longer ($p < 0.001$) than driver control (*w*^{1118}; *GAL4*$^{109(2)80}$/+, black curve). The dotted line represents the EP alone (*w*^{1118}; *EP(2)2383*/+).

(C) *GLaz* cDNA transgenic lifespan extension under normoxia, at 29ºC. *GLaz* cDNA overexpressors (*w*^{1118}; *GAL4*$^{109(2)80}$/*UAS-GLaz*^{2}, red curve) live 23% longer ($p < 0.001$) than driver control (*w*^{1118}; *GAL4*$^{109(2)80}$/+, black curve). The dotted line represents the transgene alone (*w*^{1118}; *UAS-GLaz*^{2}/+).

(D) GAL4-dependent lifespan extension under hyperoxia in the transgenic flies. GLaz cDNA overexpressors (*w*^{1118}; *GAL4*$^{109(2)80}$/*UAS-GLaz*^{2}, red curve) live 12% longer ($p < 0.001$) than the driver control (*w*^{1118}; *GAL4*$^{109(2)80}$/+, black curve). Each graph represents a trial with at least 100 flies, typical of several repeated experiments.

(Figure 2D) compared to the driver control. To test whether the overexpressor flies also had improved vigor associated with their hyperoxia resistance, we performed behavioral assays. After 4 days in hyperoxia, both vertical and horizontal locomotor performances were much better in flies overexpressing *GLaz*, as compared to controls (Figures 3A and 3B). The same genotypes, when maintained under normoxia, showed no differences.

Flies Overexpressing *Glaz* Are More Resistant to Starvation

Resistance to multiple extrinsic stressors is a hallmark of several long-lived mutants [24]. We tested flies overexpressing *GLaz* for resistance to wet or dry starvation. Overexpression of *Glaz* led to a 60% increase in lifespan under wet starvation (Figure 4A) and a 30% increase under dry starvation (Figure 4B). Given the predicted nature of the protein as a secreted lipid carrier, we checked whether the flies overexpressing *GLaz* were larger or displayed higher lipid or protein content. There was no significant difference in dry weight (Figure S3A), protein content (Figure S3B), or fat content (Figure S3C) between flies overexpressing *GLaz* and the driver crossed to *w*^{1118}.

GLaz Overexpression Protects against Behavioral Deficits Caused by Hypoxia

ApoD is upregulated in a mouse model of stroke [25]. Therefore, we tested the ability of flies that overexpress *Glaz* to withstand periods of oxygen deprivation, followed by recovery under normoxia. The flies were subjected to hypoxia in 100% N_2 for 30 min, then transferred back to a normoxic environment. After awakening, flies overexpressing *Glaz* were much more active than control flies. In our assay of vertical locomotion performance, 1 hr after their recovery, 75% of the *GLaz* overexpressors climbed to the top third of the vial, while only 10%–15% of control flies managed to do the same (Figure 4C). This difference was still evident 2 days later. Before hypoxia exposure, no difference in climbing ability was observed.

Discussion

We report that overexpression of *GLaz,* by means of independent EP insertions or transgenic lines, can result in increased resistance to stress and extension of lifespan. These results are consistent with ApoD being part of a damage-control system. The mechanisms involved in these effects remain to be determined: GLAZ, and

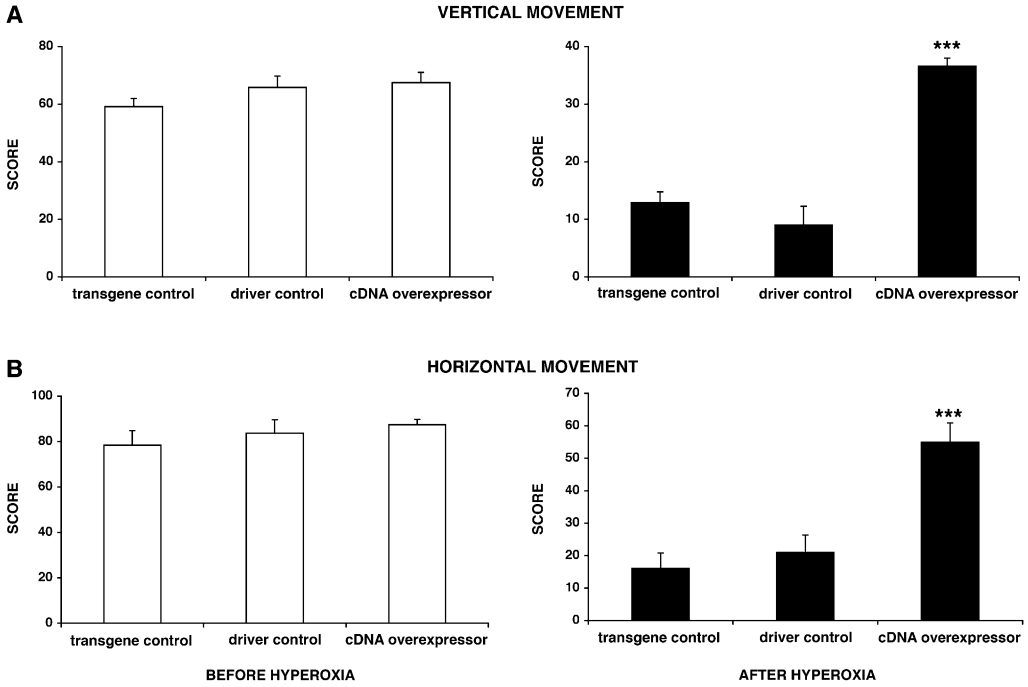

Figure 3. Overexpression of *Glaz* Protects against Hyperoxia-Induced Behavioral Decline

(A) Assay of vertical locomotion performance before (white bars) and after (black bars) 4 days under hyperoxia. After hyperoxia, flies overexpressing GLaz (w^{1118}; $GAL4^{109(2)80}$/*UAS-GLaz2*) climbed significantly better than either control (w^{1118}; $GAL4^{109(2)80}$/+ or w^{1118}; *UAS-GLaz2/+,* Student's t test, $p < 0.001$).

(B) Assay of horizontal locomotion performance before (white bars) and after (black bars) 4 days under hyperoxia. Flies overexpressing *GLaz* (w^{1118}; $GAL4^{109(2)80}$/*UAS-GLaz2*) performed significantly better than controls (w^{1118}; $GAL4^{109(2)80}$/+ or w^{1118}; *UAS-GLaz2/+,* Student's t test, $p < 0.001$). Error bars represent SEM.

perhaps APOD, may transport lipophilic molecules to and from cells, thus helping to repair damaged membranes or clear cholesterol and fatty acids released by dying cells. In mammals, the binding affinity for arachidonic acid may point to a role in the control of inflammation. They may also quench deleterious molecules such as lipid peroxides or scavenge other free radicals. In the accompanying paper, Sanchez et al. [QA] observe a depletion of lipid stores in *GLaz*-deficient mutants. GLAZ may work as part of a shuttle system, used to maintain stores at a normal level, while providing support to other tissues in situations of stress.

We screened multiple drivers, with multiple responder lines, and could not point to a single tissue as a necessary or sufficient site of upregulation to produce increased longevity. The amount of overexpression of such a secreted protein may matter more than its site of production. Furthermore, it must be kept in mind that the expression patterns of many drivers change during development and with age and that *GLaz* is likely to be secreted. Interestingly, overexpression with strong neuronal drivers did not extend adult lifespan. In the accompanying paper, Sanchez et al. [QA] report that normal adult expression of *GLaz* is primarily in glial cells, and to some extent in the cardia and hemocytes. In embryos, the transcript is present in the nervous system [11] and the developing gut. In the embryonic nervous system, *Glaz* is expressed in the longitudinal glia. These cells are known to be crucial for axon guidance and neuronal trophic support and can be considered homologous to oligodendrocytes of vertebrates. Originally, the grasshopper homolog, *Lazarillo,* was found to be important for axon guidance [26]. One of our most effective drivers, $GAL4^{109(2)80}$, is expressed in adult vertical flight muscles, and, to a lesser extent, in the adult nervous system, but has a very restricted expression pattern during development. It is therefore possible that strong early overexpression in neurons has adverse effects that mask the benefits of GLAZ in the adult, where it may circulate in the hemolymph to reach its target tissues.

Flies overexpressing *Glaz* fared much better in behavioral tests after hyperoxia exposure. We have recently shown that the flight muscle is one of the earliest tissues to suffer damage under hyperoxia and that old flies also display some of the alterations seen under hyperoxic stress [27]. It is conceivable that overexpression of *GLaz* in the flight muscles (via $GAL4^{109(2)80}$) is beneficial either by improving nutrient exchange or by delaying cellular damage. In the mouse, ApoD is upregulated in response to cerebral stroke [25]. Some of the common therapies for victims of such damage aim at lowering the overall metabolism of the affected area to decrease the oxidative burden on cells. We show that *GLaz* overexpression can protect against behavioral decline induced by exposure to hypoxia. *GLaz* overexpression might make the flies more resistant to the cellular insult of the stresses studied here, thus hastening recovery.

In recent years, *Drosophila* has become a powerful model for studying neurodegenerative disorders [28]. Understanding how lipocalins regulate lifespan or

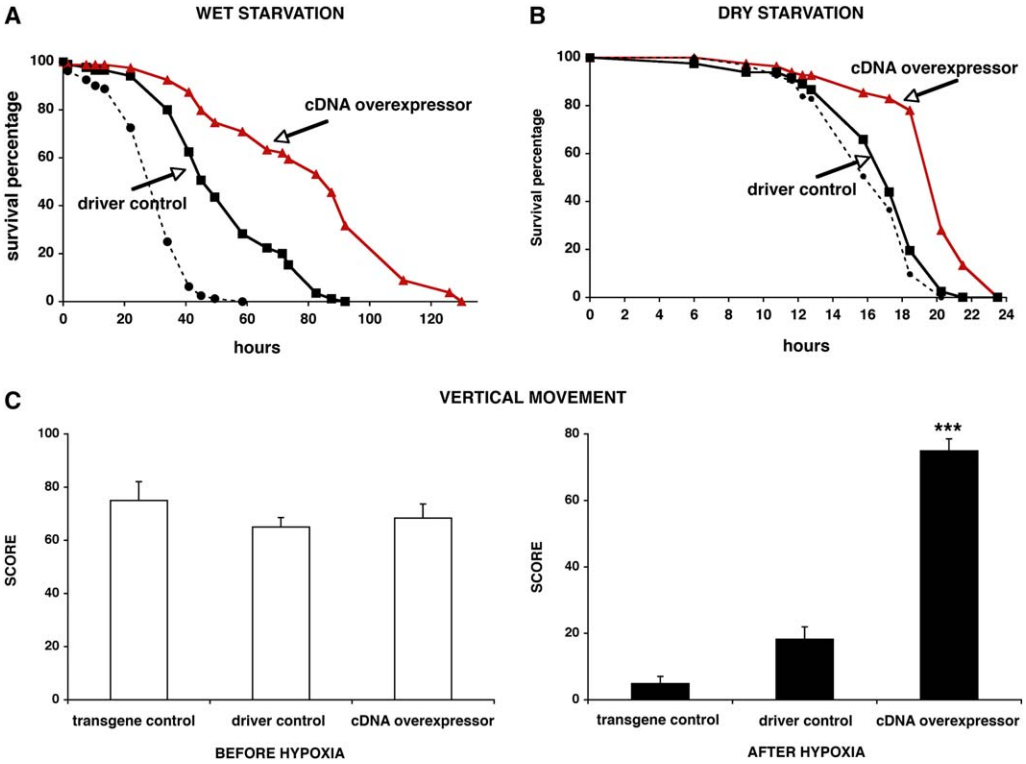

Figure 4. Overexpression of *Glaz* Increases Resistance to Multiple Extrinsic Stresses

(A) Wet starvation. Flies overexpressing GLaz (w^{1118}; $GAL4^{109(2)80}$/*UAS-GLaz2*, red curve) lived 60% longer than driver controls (w^{1118}; $GAL4^{109(2)80}$/+, $p < 0.001$).

(B) Dry starvation (desiccation). Flies overexpressing *GLaz* (w^{1118}; $GAL4^{109(2)80}$/*UAS-GLaz2*) lived 30% longer than driver controls (w^{1118}; $GAL4^{109(2)80}$/+, $p < 0.001$). Dotted line represent the transgene alone (w^{1118}; *UAS-GLaz2*/+). Each graph represents a trial with at least 100 flies, typical of several repeated experiments.

(C) Hypoxia. Assay of vertical locomotion performance after 30 min under hypoxia, followed by 1 hr recovery under normoxia. White bars represent performance before hypoxia exposure, with no significant difference between genotypes. Black bars represent performance after hypoxia exposure and recovery: flies overexpressing GLaz (w^{1118}; $GAL4^{109(2)80}$/*UAS-GLaz2*) climbed significantly better (Student's t test, $p < 0.001$) than either controls (w^{1118}; $GAL4^{109(2)80}$/+ or w^{1118}; *UAS-GLaz2*/+). Error bars represent SEM.

rescue neurological disorders in *Drosophila* may help to understand the role of ApoD in the human brain and suggest ways of manipulating its expression and function, providing clues to potential therapeutic routes.

Supplemental Data

Supplemental Data include three figures and Supplemental Experimental Procedures and can be found with this article online at http://www.current-biology.com/cgi/content/full/16/7/674/DC1/.

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