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The *Heat Shock Protein 26* Gene Is Required for Ethanol Tolerance in *Drosophila*

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Abstract: Stress plays an important role in drug- and addiction-related behaviours. However, the mechanisms underlying these behavioural responses are still poorly understood. In the light of recent reports that show consistent regulation of many genes encoding stress proteins including heat shock proteins following ethanol exposure in *Drosophila*, it was hypothesised that transition to alcohol dependence may involve the dysregulation of the circuits that mediate behavioural responses to stressors. Thus, behavioural genetic methodologies were used to investigate the role of the *Drosophila hsp26* gene, a small heat shock protein coding gene which is induced in response to various stresses, in the development of rapid tolerance to ethanol sedation. Rapid tolerance was quantified as the percentage difference in the mean sedation times between the second and first ethanol exposure. Two independently isolated P-element mutations near the *hsp26* gene eliminated the capacity for tolerance. In addition, RNAi-mediated functional knockdown of *hsp26* expression in the glial cells and the whole nervous system also caused a defect in tolerance development. The rapid tolerance phenotype of the *hsp26* mutants was rescued by the expression of the wild-type *hsp26* gene in the nervous system. None of these manipulations of the *hsp26* gene caused changes in the rate of ethanol absorption. Hsp26 genes are evolutionary conserved, thus the role of *hsp26* in ethanol tolerance may present a new direction for research into alcohol dependency.

Keywords: alcohol, tolerance, Hsp26, glial cells, *Drosophila*

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Introduction

Alcohol consumption in humans causes long-term physiological changes including tolerance. Tolerance in this sense can develop to both the pleasurable (eg, euphoria and loss of social inhibition) and the aversive (eg, loss of motor coordination and sedation) effects of ethanol,¹ and is thought to encourage increased alcohol intake, development of physical dependence and addiction.² Indeed, alcoholics acquire extra-ordinary tolerance to the intoxicating effects of ethanol, usually associated with dependence and uncontrolled craving to continue drinking.³

Tolerance is defined as a reduction in drug responsiveness seen after repeated exposures to that drug. There are believed to be different types of tolerance: Acute tolerance, which occurs within drug session/experience; rapid tolerance, which occurs after the completion of a single drug exposure/experience; and chronic tolerance which arises from serial drug exposures.⁴ Further, two mechanisms of tolerance which are not mutually exclusive have been reported: metabolic/pharmacokinetic tolerance, involving changes in the disposition of ethanol (such as absorption, excretion or metabolism) leading to efficient removal of alcohol from the body and functional/pharmacodynamic tolerance involving changes experienced at a cellular level and mediated by adaptations in neural function.¹

Drosophila has been shown to acquire rapid tolerance to the sedating effects of ethanol,⁵ and in flies many genes have been implicated in ethanol tolerance. For example, flies carrying a mutation in the *hangover* (*hang*) gene (a gene encoding a nucleic acid binding zinc finger protein) were implicated in the development of reduced ethanol tolerance as were flies lacking the neuromodulator octopamine owing to a mutation in the gene encoding tyramine β hydroxylase (*Tbh*).^{5,6} Further, induction of tolerance was completely abolished in flies simultaneously carrying null mutations in *hang* and in *Tbh*, indicating that both genes are involved in different pathways in the induction of ethanol tolerance.⁶ Flies carrying *slowpoke*, a Ca^{2+} -activated K^{+} channel gene which is a critical modulator of neuronal excitability have also been shown to be required for the acquisition of tolerance to ethanol.^{7,8} The *Drosophila* homolog of JWA, a microtubule binding protein, was also reported to be necessary for the acquisition of

ethanol tolerance in *Drosophila*.⁹ Finally, a study of flies defective in long-term memory implicated several genes in reduced ethanol tolerance including *exba* (*elF-5C*), a translational regulator involved in axon guidance); *pumilio* (translational regulator) and *formin3*, involved in actin assembly.¹⁰

Microarray experiments have been recently used to profile changes in gene expression following acute ethanol exposure in *Drosophila*.^{11–13} One of these changes involves the regulation of genes encoding several heat shock proteins (Hsp). Interestingly, one of the most strongly regulated Hsp genes in a recent study of acute ethanol regulation of gene expression in *Drosophila* was *hsp26*,¹³ a member of the small heat shock proteins (sHsp) that function as molecular chaperones responsible for the prevention of non-native protein aggregation during protein folding/refolding, and facilitation of proteolysis of damaged proteins.¹⁴

Hsp26 is a member of a conserved protein family containing 2 domains: the highly conserved C-terminal region and an N-terminal domain which exhibits sequence divergence ranging from 12 to 40 kDa in different organisms.¹⁵ Unlike the higher molecular weight Hsps, the binding and release of substrates in Hsp26 does not require either ATP binding or ATP hydrolysis.¹⁶ Hsp26 has been shown to be a temperature regulated chaperone in yeast¹⁵ and it is involved in aging and oxidative stress in *Drosophila*.^{17,18} *Drosophila hsp26* has been shown to be expressed in the neurocytes of the brain and the thoracic ganglion.¹⁹ Hsp26 is transcribed during certain developmental stages in the absence of heat shock²⁰ and is expressed with a robust circadian rhythm in the adult *Drosophila* head,²¹ indicating a role in sleep homeostasis.

To determine whether the *hsp26* gene mediates some aspects of the behavioural adaptations underlying the ethanol response in *Drosophila*, flies carrying mutations in this gene were tested for ethanol sensitivity and tolerance. Here, we show that whereas flies carrying P-element mutations near the *hsp26* gene showed normal ethanol sensitivity, they displayed virtually abolished tolerance to ethanol sedation. The ethanol tolerance defect was also seen when *hsp26* gene expression was silenced in glia and the nervous system. Pan-neuronal expression of wild-type *hsp26* was also shown to rescue this ethanol tolerance phenotype. Thus, this study has indicated

a new role for this member of the small heat shock protein family as an important element in mediating ethanol tolerance.

Materials and Methods

Fly stocks

The wild type strain used in this study was *w⁺*; *Iso2C*; *Iso 3I* isogenised on the second and third chromosomes and reported to behave similarly to the commonly used Canton-S stock in a range of behavioural tests,²² and kindly provided by Cahir O' Kane (University of Cambridge, Cambridge, UK). Mutant strains and P[GAL4] lines used in this study were *hsp26^{EY10556}*, *hsp26^{KG02086}* repo-GAL4 (7415) (chromosome III),²³ elav-GAL4 (III) (8760) and elav-GAL4 (11) (8765) from the Bloomington *Drosophila* stock centre at Indiana University (Bloomington, IN). UAS-*hsp26* (II)¹⁷ flies were obtained from Horng-Dar Wang (National Tsing Hua University, Taiwan) and *Tbh^{nm18}* flies from Maria Monastirioti (Institute of Molecular Biology and Biotechnology (IMBB), Greece). The UAS-*hsp26^{RNAi}* was line v6983 (III) from the Vienna *Drosophila* RNAi centre. The P[GAL4 lines] were verified for expression in the glia or neurons of the adult *Drosophila* brain by confocal imaging of the adult brain of flies carrying repo-GAL4 and UAS-GFP or elav-GAL4 and UAS-GFP (Supplementary Fig. 1 and data not shown). Flies were raised on standard maize meal food at 18 °C.

Sedation assay

The sedation assay previously described by Wen et al²⁴ was modified and used to measure ethanol sensitivity in *Drosophila*. For this assay, 20 active and well fed males (or females as otherwise indicated) were used for each trial. These flies were selected under CO₂ anaesthesia and allowed to recover for 24 h before use. 1 ml ethanol solution at 50% concentration was added to a piece of folded Kimwipe tissue (11.4 × 21.5 cm) with edges sealed by transparent tape and laid at the bottom of a 180 ml plastic fly bottle. Flies were then transferred immediately into the bottle and the bottle sealed with a paper lid and parafilm. The active flies remained at the top of the bottle and the sedated flies that dropped to the bottom were counted at 6-min intervals. Counting started immediately flies were introduced into the bottles. The percentage active flies for each time interval of

6 minutes was calculated as the number of flies active at a given time divided by the total number of flies and multiplied by 100 as given by this formula: % Active Flies = $x_t / N \times 100$ where x_t is the number of active flies at a given sedation time, t and N the total number of sample flies. The Mean Sedation Time (MST) used as a measure of the resistance to the sedative effects of ethanol was calculated as the sum of the number of flies sedated at every 6 minutes multiplied by the time of sedation in minute and divided by the total number of flies sedated as given by this equation:

$MST = \sum x_t \times t / N$ where x_t is the number of flies sedated at a given sedation time t and N the total number of flies sedated.

Tolerance assay

Sedation assays measuring rapid tolerance were performed essentially as in the sedation assay for ethanol sensitivity but after initial exposure (MST1), flies were collected in vials and allowed to recover in a humidified room at 18 °C on fresh food. They were then exposed to ethanol for a second time. The second exposure (MST2) was initiated exactly 4 h after the start of the first exposure. Tolerance development (ie, percentage increase in tolerance) was calculated relative to the MST of flies following their first and second exposure in the sedation paradigm, using the formula (MST2 – MST1/MST1 × 100).

Heat-shock-ethanol cross-tolerance assay

Flies were incubated at 38 °C for 3 min in a vial containing fresh food that had been pre-incubated at 38 °C for 18 hrs. After a recovery period of 4 h at 18 °C, the flies were exposed to ethanol in the sedation paradigm (MST^{hs+}). Tolerance was calculated with respect to flies that were not heat-treated (MST^{hs-}), using the formula (MST^{hs+} – MST^{hs-}/MST^{hs-} × 100).

Rescue experiments

P[UAS-*hsp26*] and P[GAL4-elav]/Cyo transgenic lines were crossed into the *hsp26^{EY10556}* mutant background to generate P[UAS-*hsp26*]/P[UAS-*hsp26^{EY10556}*]/*hsp26^{EY10556}* and P[GAL4-elav]/Cyo; *hsp26^{EY10556}*/P[UAS-*hsp26^{EY10556}*] flies. For rescue experiments, male flies were F1 progeny of a cross between *hsp26^{EY10556}* flies carrying a P[GAL4-elav] driver and UAS-*hsp26*; *hsp26^{EY10556}* flies.

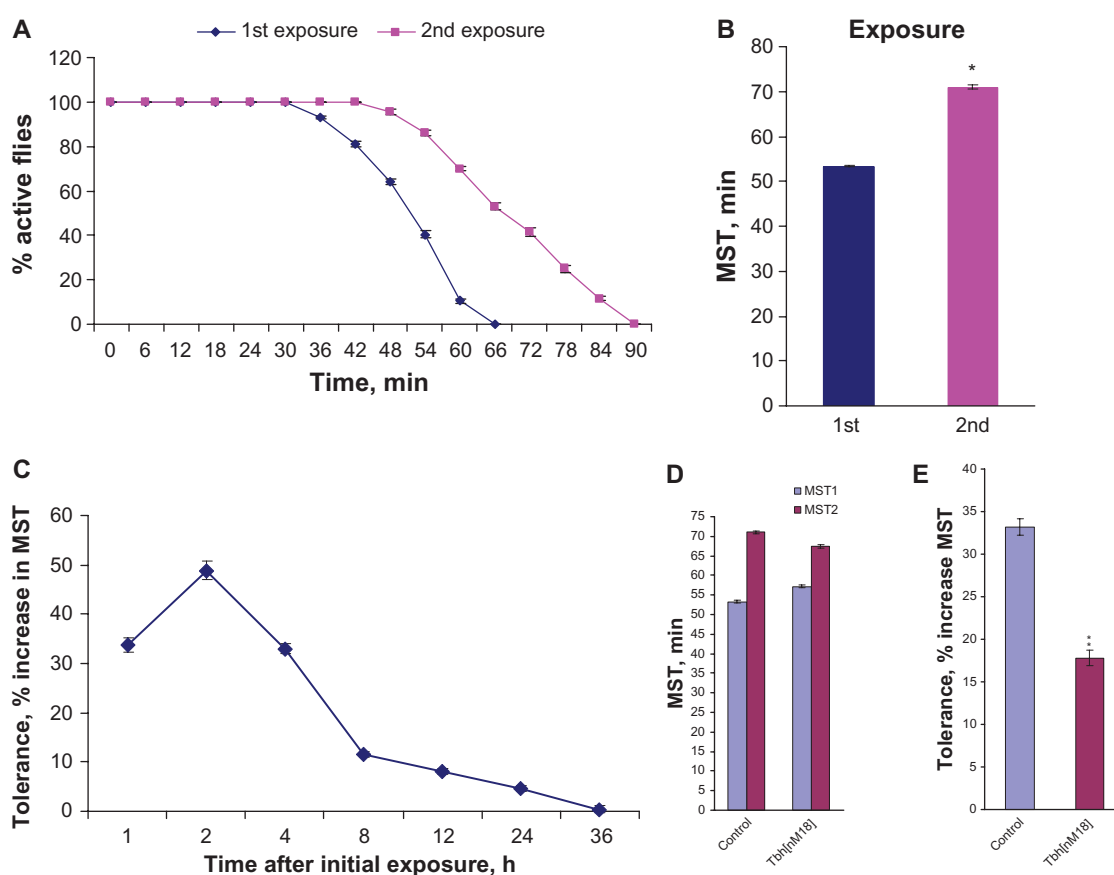


Figure 1. Ethanol tolerance measured in the sedation assay. The sensitivity to ethanol vapour of a population of 20 flies was measured in the sedation assay, a simple but highly efficient technique that measures the duration of fly activity after exposure to a sedative dose of ethanol. At 50% standard ethanol concentration, wild-type flies become sedated over a period of about an hour (**A**) with a mean sedation time (MST) of 53.3 ± 0.3 min (**B**). When these flies were reintroduced into the vial 4 h after the first exposure, their sedation profile shifted to the right (**A**) and their new MST was 71.0 ± 0.4 min (**B**), which corresponds to a $33.0 \pm 1.0\%$ increase in resistance, $N = 20$, $P < 0.0001$. **C**) Kinetics of tolerance acquisition and decay, showing the percent increase in mean sedation time (MST) between the second and the first exposures, $N = 20$ (0 h and 4 h); $n = 6-10$ (all other time points). **D** and **E**) *Tbh* flies acquire some tolerance to ethanol (**D**) but much less than wild-type flies (**E**). $n = 5$, $P < 0.00001$.

Notes: Error bars represent standard error of the mean (s.e.m). In all figures N or n corresponds to the number of experiments, not the number of flies.

Ethanol absorption and metabolism

Ethanol concentrations were determined from whole fly homogenates of 2 flies per sample using an Analox Instruments (London, UK) ethanol analyser (Alcometer). To study ethanol absorption, flies were exposed to 100% ethanol vapour for 12 min in a recovery assay as previously described²⁴ and culled at 0, 1, 2, 3 or 4 h recovery time after exposure. Immediately after each recovery period flies were frozen in dry ice and homogenised in 20 μ l PBS buffer, pH 7.4. The homogenate was then centrifuged at 12,000 rpm at 4 °C for 10 min, and the supernatant was collected. The ethanol concentration in the supernatant was measured using the Alcometer.

Statistical analysis

Statistical significance was assessed by either Student's paired t-tests assuming equal variance or one-way

analysis of variance (ANOVA) with Newman-Keuls post-hoc tests.

Results

Rapid tolerance can be measured in the sedation assay

It has previously been reported that adult *Drosophila* develops tolerance after a single ethanol exposure.⁵ Flies upon exposure to ethanol vapour become hyperactive, lose postural control and eventually became sedated.²⁵ The sensitivity of a population of flies to ethanol can be measured using the sedation assay, a paradigm that quantifies ethanol-induced sedative effects.²⁴ Briefly, flies are introduced into a vial with a swab soaked with ethanol of a particular concentration. As flies became intoxicated with ethanol, they display hyperactivity before falling to the

bottom of the vial and later become sedated. At 50% ethanol concentration (the standard ethanol concentration used in this study) wild-type flies reproducibly become sedated with a mean sedation time of 53.3 ± 0.3 min (Fig. 1B).

To determine whether rapid tolerance could be measured in the sedation assay, flies initially tested in the sedation assay as previously described, were collected in vials containing fresh food and allowed to recover in a humidified room at 18 °C before re-testing in the sedation paradigm. The second exposure in the sedation assay (MST2) was initiated exactly 4 h after the start of the first exposure. The 4 h interval was chosen not only to ensure that flies had fully recovered from the sedative effect of ethanol but also to ensure they had completely metabolised all ethanol absorbed and had had time to rehydrate and feed.⁵ Tolerance was calculated based on the percentage difference in MST between these two exposures (Figs. 1A, B). It was found that wild-type flies were more resistant to the second exposure to ethanol displaying a MST of 71 ± 4 min, an increase of nearly 18 min from the MST of their first exposure. In this case, tolerance is defined as the relative increase in MST between the first and the second exposure, which for wild-type flies correspond to 33% under the standard experimental conditions.

A previous study using the inebriometer⁵ has shown that flies develop rapid tolerance with kinetics of dissipation that was biphasic. To determine the kinetics of tolerance decay using the sedation assay protocol, flies were exposed in the sedation vial twice at a various time intervals and their tolerance acquisition quantified at the appropriate time (Fig. 1C). Consistent with a previous characterisation of rapid tolerance using the inebriometer, it was observed that the kinetics of tolerance dissipation was biphasic, suggesting the involvement of two different processes or mechanisms. Maximal tolerance, a $48.8\% \pm 1.9\%$ increase in MST, was achieved within a 2 h interval. Tolerance then decreased quickly, reaching ~12% after 8 h. It then decayed more slowly and was still detectable 24 h after the first exposure, but had disappeared by 36 h (Fig. 1C). Thus, rapid tolerance measured with the sedation assay is very similarly to that previously measured with the inebriometer, an assay that measures the fly's postural control on exposure an intoxicating dose of ethanol.

To validate this present assay *Tbh^{nm18}* mutant flies defective for the octopamine biosynthetic enzyme Tbh and previously reported to display impaired rapid tolerance development using the inebriometer assay⁵ were tested for rapid ethanol tolerance in the tolerance paradigm. Flies were pre-tested in the sedation assay and were then re-assayed in the same paradigm after 4 h and rapid tolerance was quantified (Fig. 1D). As previously described, the *Tbh^{nm18}* mutant showed a marked decrease in ethanol tolerance compared to the wild type control (Fig. 1E), thus validating the tolerance protocol used in this study. This indicates that rapid tolerance, as measured by the sedation and inebriometer assays develops, in part, a common mechanism.

Mutations affecting the *hsp26* gene abolish ethanol tolerance

A recent microarray study has shown that the *hsp26* gene was strongly upregulated from 0 to 4 h following acute ethanol exposure in *Drosophila*.¹³ Over this time period, the levels of many genes increase and then fall to base-line levels suggesting that they may mediate acute effects of ethanol. Given that the expression level of *hsp26* differed from the base line at the end of the 4 h time frame, we wondered if this gene was implicated in ethanol tolerance in *Drosophila*.

To ascertain a possible role for the *hsp26* gene in ethanol tolerance, two mutant alleles carrying different P-element insertions in the same position near the *hsp26* gene were tested in the tolerance paradigm. Whereas the ethanol sensitivities of these alleles were normal in the sedation assay, these flies displayed virtually abolished tolerance when tested 4 h after the initial ethanol exposure (Figs. 2A, B). The behaviour of these alleles cannot be explained by an alteration in the kinetics of tolerance acquisition as a similar marked defect in tolerance was observed when these two alleles were exposed to ethanol using protocols earlier shown to produce maximal tolerance in the sedation assay (Figs. 2C, D). Both male and female *hsp26^{EY10556}* flies behaved in the same manner. Male flies heterozygous for the *hsp26^{EY10556}* mutant were also tested for development of rapid tolerance. The *hsp26^{EY10556}* heterozygous males displayed ethanol tolerance that is 75% of wild-type levels suggesting that tolerance is sensitive to the dosage of *hsp26*. Thus, the two independently isolated *hsp26* alleles in

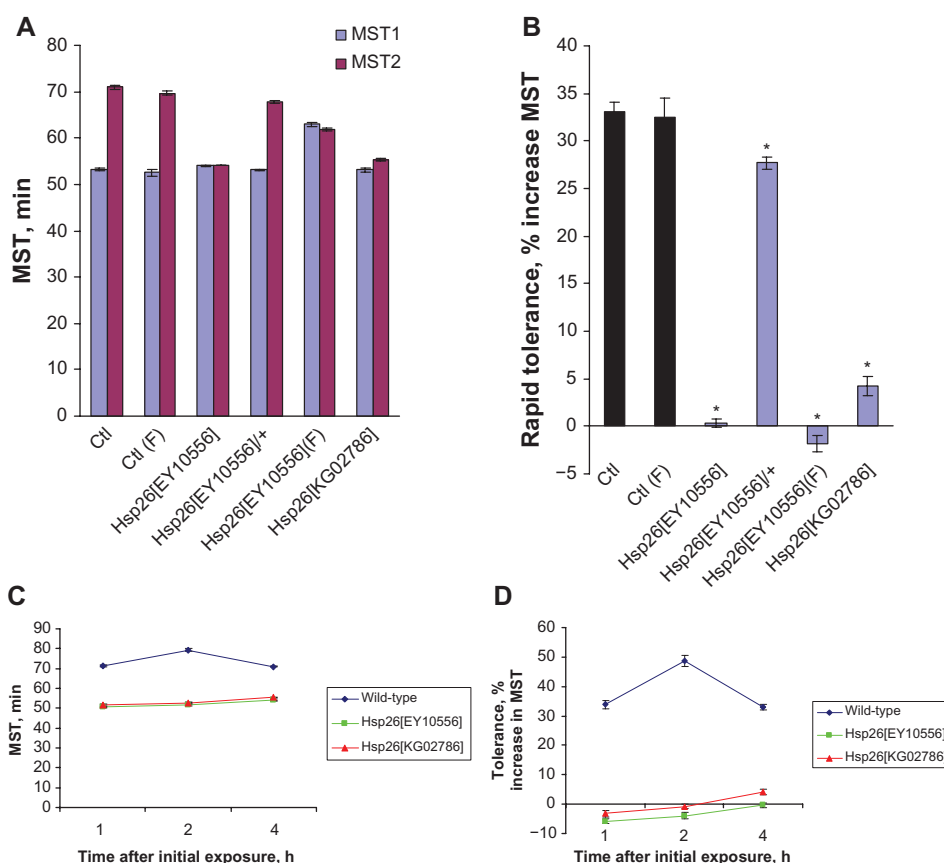


Figure 2. Tolerance is almost completely abolished in *hsp26* alleles. **A** and **B**) *hsp26^{EY10556}* and *hsp26^{KG02786}* flies do not show a statistically significant increase between 1st and 2nd exposure to ethanol (**A**) and thus no increase in tolerance (**B**), $n = 6$, $*P < 0.0001$. **C** and **D**) kinetics of tolerance acquisition and decay of *hsp26* and wild-type flies. Flies were exposed to ethanol once and then tested in the sedation assay after the three time intervals shown. Tolerance was calculated for each set of flies as the percent increase in mean sedation time (MST) between the second and the first exposures, $N = 6-20$ (0 h and 4 h); $n = 4-10$ (1 h and 2 h).

Notes: Error bars represents s.e.m. Male flies were used unless indicated (F).

their homozygous state exhibited very similar ethanol related behaviours: both showed normal sensitivity and virtually abolished ethanol tolerance development. This indicates the specificity of this behaviour to the *hsp26* gene and also rules out any effects due to genetic background at least in the homozygous state.

Exposure to an acute dose of ethanol induces a stress response.²⁶ Previous work⁶ has indicated that the stress pathway(s) induced by ethanol are linked to, or overlap with, stress pathways induced by other stressors. Hsp26 encodes a cytoplasmic protein involved in the response to several forms of stress, including heat shock.²⁷ To assess whether prior heat shock of flies might induce ethanol tolerance in the sedation assay, and determine the effect of a heat pulse on the tolerance defect seen in the *hsp26* alleles, both the wild-type control and *hsp26* flies were tested for ethanol tolerance using the heat shock

protocol (see Methods). Heat exposure of wild-type flies (38 °C for 5 min) led to a 42% increase in MST when measured in the sedation assay 4 h later. However, the two *hsp26* alleles displayed a 10% and 9% increase in MST when treated with the same protocol. Thus, two *hsp26* genotypes displayed a substantially reduced level of tolerance compared with the control flies ($P < 0.001$, Fig. 3). The fact that *hsp26* alleles are deficient in the acquisition of tolerance in both protocols indicates that the tolerance produced by ethanol and heat shares some common mechanism(s). *hsp26* flies have some capacity for developing tolerance after heat shock, suggesting that other pathways are also involved.

Ethanol absorption and metabolism of *hsp26^{EY10556}*

One possible explanation for the altered ethanol phenotypes observed for the *hsp26* mutants tested

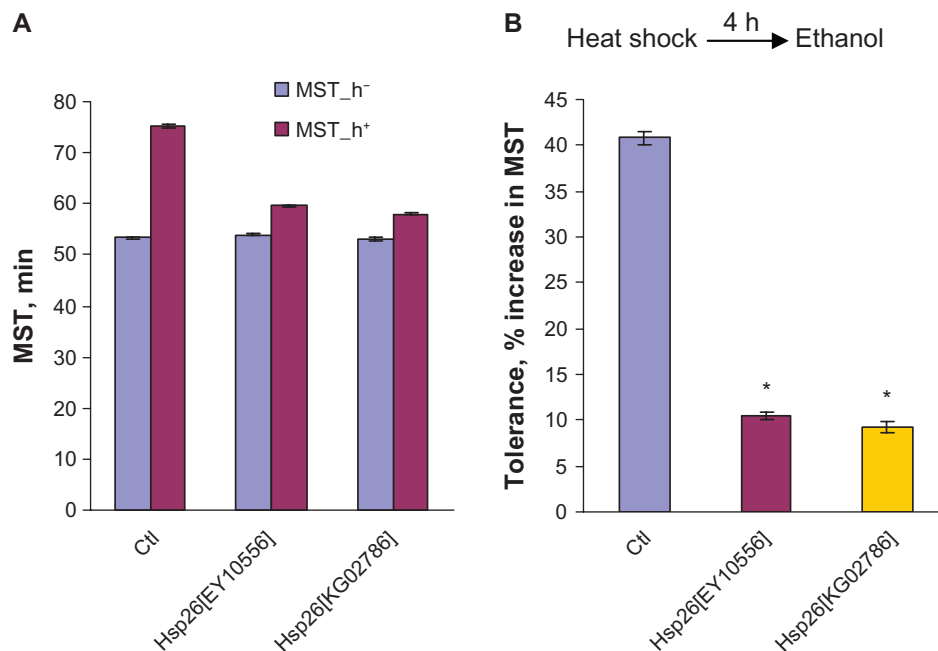


Figure 3. Heat-ethanol cross-tolerance of wild-type and *hsp26* flies. **A)** *hsp26* flies are indistinguishable from wild-type control in the first ethanol exposure before the heat shock. **B)** *hsp26* mutant flies however showed significantly reduced tolerance development in the sedation assay following heat pulse exposure compared to the control (wild-type) flies.

Notes: $n = 6$, $*P < 0.0001$. MST_{h⁻} and MST_{h⁺} denote MST obtained from flies without and after heat shock respectively. Error bars represent s.e.m.

in this study is that the modulation of expression of the *hsp26* gene is an adaptive response of fly nervous system to compensate for at least some of the sedating effects of ethanol, and this change in expression is at least partially responsible for the altered rapid tolerance to ethanol. However, it is also possible that these mutants exhibit altered ethanol phenotypes as a result of differences in their ethanol absorption and/or metabolism when compared to a wild-type strain.

To determine whether there was altered ethanol metabolism in flies, the ethanol concentration in fly extracts prepared from *hsp26*^{EY10556} and wild-type flies exposed to constant ethanol vapour (100%) for 12 min and allowed to recover for 0, 1, 2, 3, or 4 h was measured. The ethanol concentration in extracts of *hsp26*^{EY10556} flies was indistinguishable from that of the wild-type control flies at all time points (Fig. 4). As shown in the Fig. 4, the results reveal a very similar rate of ethanol clearance for the two genotypes. Therefore, the altered ethanol phenotypes of these mutants were more likely due to pharmacodynamic changes, and not pharmacokinetic changes. The results also give an ethanol content of about 44 mM in wild-type flies immediately after sedation. This corresponds to about 0.20% in human blood alcohol concentration

(BAC) which causes severe loss of the response to sensory stimuli and loss of consciousness.²⁸

Neuronal expression of Hsp26 on ethanol sedation

The results described above implicate the *hsp26* gene in the regulation of ethanol-induced behaviour: *hsp26* mutations affect tolerance but not sensitivity of flies to ethanol in the sedation assay. To determine whether *hsp26* mediates this behaviour by acting within the nervous system, we used the GAL4/UAS gene expression system²⁹ to target the expression of the *hsp26* gene to the nervous system.

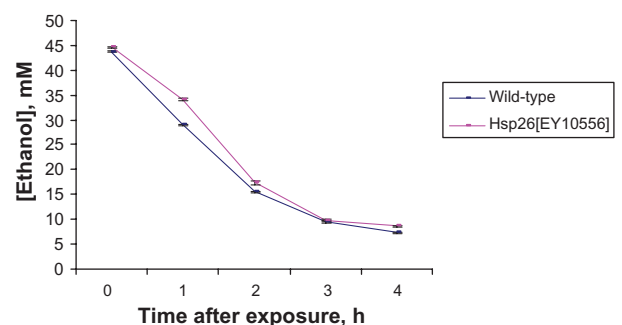


Figure 4. Ethanol concentrations in flies after ethanol exposure. No significant differences were seen between the *hsp26*^{EY10556} mutant and the wild-type control (Student's *t*-test; $n = 3$).

First, to determine whether silencing of Hsp26 expression in neurons can mimic the normal ethanol sensitivity phenotype observed in the *hsp26* mutant flies, we used UAS-*hsp26*^{RNAi} to knockdown the expression of the *hsp26* gene in the nervous system of wild-type flies using elav-GAL4 driver, which drives expression in all *Drosophila* neurons. Flies carrying the elav-GAL4 driver and a P[UAS-*hsp26*^{RNAi}] were generated and tested for ethanol sensitivity in the sedation assay. As with the results from *hsp26* mutants, pan-neuronal silencing of the *hsp26* gene in all neurons did not result in any significant alterations in ethanol sensitivity (Fig. 5A blue bar). We also determined whether an increase in the endogenous levels of Hsp26 expression in all neurons alters the

behavioural response to ethanol. Thus, flies carrying one wild type copy each of the UAS-*hsp26* transgene and elav-GAL4 were tested for ethanol sensitivity in the sedation assay. Overexpression of Hsp26 in the nervous systems led to resistance to the sedating effects of ethanol (Fig. 5C blue bar). The overexpression results correlate with the microarray data presented by Kong and colleagues.¹³ When flies were exposed to ethanol, transcription of *hsp26* rose. This response may reflect a protective function of the gene and probably buffers the cell from further insults, hence, causing the reduced ethanol sensitivity of flies overexpressing this gene in the nervous system.

In addition, we also determined whether silencing of Hsp26 expression in the nervous system can mimic

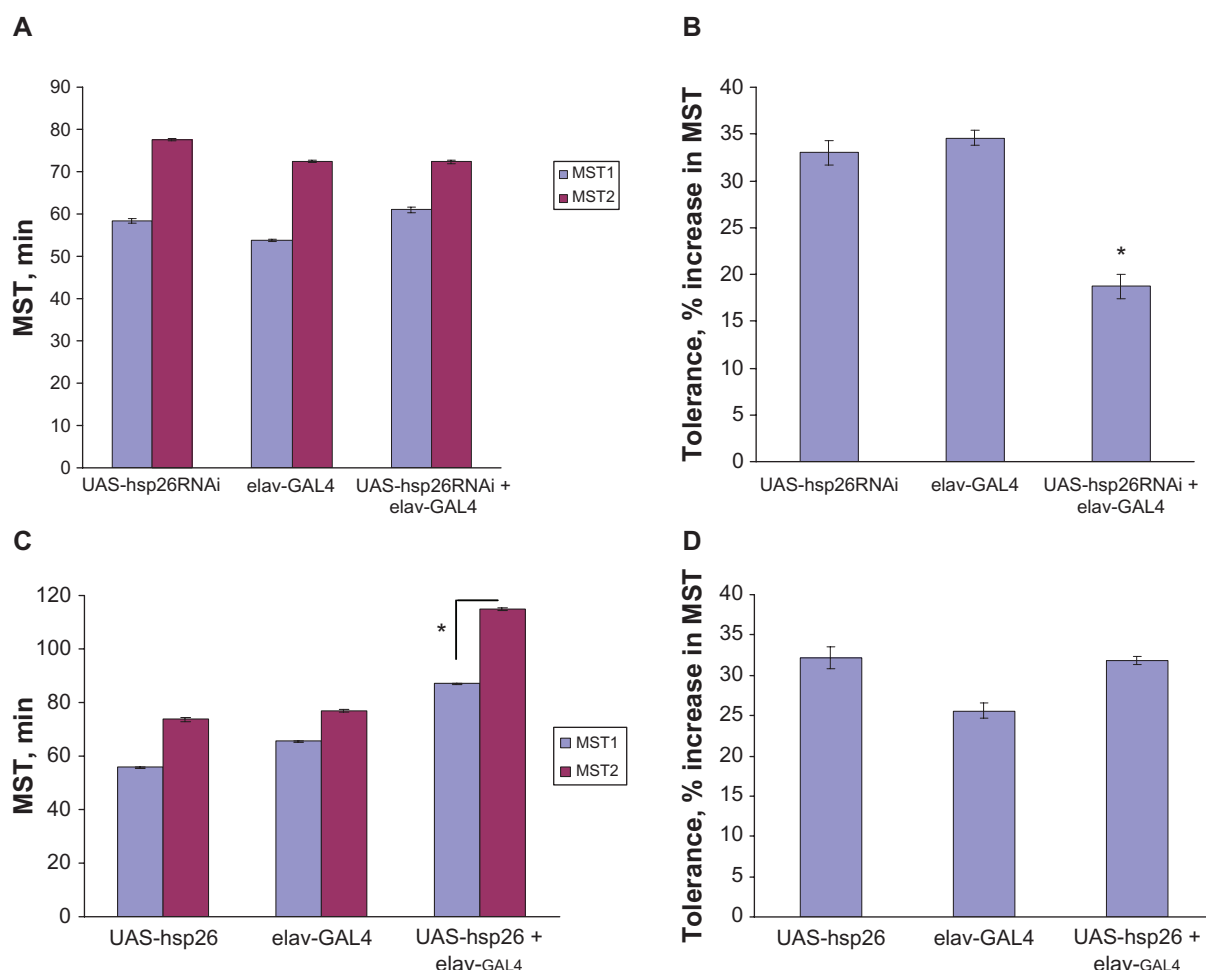


Figure 5. Hsp26^{RNAi} expression in the nervous system reduced tolerance. Upper and lower left panel (A, C) show the mean sedation time (MST) from the sedation assay of naïve flies (first exposure, blue bars) and flies pre-exposed to ethanol (second exposure, purple bars). Upper and lower right panel (B, D) show the development of ethanol tolerance, expressed as a percentage increase in MST between the two exposures. **B)** *hsp26*^{RNAi} expression when driven by elav-GAL4 shows significantly reduced ethanol tolerance compared with either transgene alone. **C, D)** Conversely, increased expression of *hsp26* in the nervous system using elav-GAL4 decreased sensitivity to ethanol in the first and second exposure to ethanol (C) but did not alter ethanol tolerance (D).

Notes: **P* < 0.001; *n* = 5 experiments. In all panels, error bars represent s.e.m.

the ethanol tolerance phenotypes observed in the *hsp26* mutant flies using the GAL4/UAS gene expression system as described above. As expected, pan-neuronal silencing of the *hsp26* gene results in reduced ethanol tolerance from 33.0 ± 1.3 (UAS-*hsp26*^{RNAi}) or 34.6 ± 0.8 (elav-GAL4) to 18.7 ± 1.3 (UAS-*hsp26*^{RNAi} + elav-GAL4) (Fig. 5B). Neither the elav-GAL4 driver nor the P [UAS-*hsp26*^{RNAi}] alone displayed reduced ethanol tolerance, indicating that the tolerance defect observed required the presence of both of these drivers. However, mutations in *hsp26* were previously shown to completely abolish the development of tolerance in flies. One interpretation for these results is that the silencing activity of *hsp26*^{RNAi} did not completely eliminate *hsp26* gene activity and the residual *hsp26* gene expression resulted in the small level of tolerance seen, suggesting that the acquisition of rapid tolerance is sensitive to levels of Hsp26. Consistent with this is the observation that one wild-type copy of *hsp26* gene is sufficient to increase tolerance to 75% of wild-type levels (Fig. 2B).

Although reduced levels of Hsp26 reduce rapid tolerance, increased levels of this protein have no effect when elav-GAL4 was used to drive *hsp26* in neurons in a wild-type background (Fig. 5D). The overexpression of *hsp26* in the nervous system did not alter rapid ethanol tolerance when compared to the elav-GAL4 driver or the P[UAS-*hsp26*] transgene alone (Fig. 5D). This suggests that unlike sensitivity to sedation, ethanol tolerance was not affected by raised Hsp26 levels in the nervous system.

Pan-neuronal expression rescues the *hsp26*^{EY10556} ethanol tolerance phenotype

To further confirm the role of the *hsp26* gene in the development of ethanol tolerance, the ability of a *hsp26* transgene to rescue the tolerance defect seen in *hsp26*^{EY10556} was examined. elav-GAL4/UAS-*hsp26*; *hsp26*^{EY10556} flies were tested for ethanol tolerance. Pan neuronal expression of wild-type *hsp26* restored the ethanol tolerance of *hsp26*^{EY10556} flies to wild-type control levels (Fig. 6A, B). The MST of the wild-type flies was not significantly different from the MST of the elav-GAL4; *hsp26*^{EY10556}/UAS-*hsp26*; *hsp26*^{EY10556} flies. Neither the elav-GAL4 driver nor the P[UAS-*hsp26*] transgene alone altered the ethanol tolerance of the *hsp26*^{EY10556} mutant flies substantially (Fig. 6B).

Taken together, both of these experiments show that *hsp26* activity is required for rapid ethanol tolerance and that *hsp26* regulates rapid ethanol tolerance by acting within the nervous system.

Although expression of *hsp26* in neurons fully rescues the tolerance defect, there is a possibility that glia are also involved in this process. Silencing the expression of *hsp26* in glia, by driving the expression of *hsp26*^{RNAi} with repo-GAL4, a glia-specific construct, led to a reduced tolerance phenotype (Figs. 7A, B). This raises the possibility that glia partially mediate ethanol tolerance. In contrast, to determine whether increased expression of endogenous Hsp26 in the glial cells also alters ethanol tolerance behaviour, repo-GAL4/UAS-*hsp26* flies, in a wild-type background were also tested for rapid ethanol tolerance. The overexpression of *hsp26* in the glial cells did not alter rapid ethanol tolerance (Figs. 7C, D) when compared to repo-GAL4 driver or the P[UAS-*hsp26*] transgene alone suggesting that ethanol tolerance was not affected by raised Hsp26 levels in the glia cells.

Discussion

The *Drosophila hsp26* gene is a member of small heat shock protein family that is induced by a variety of stressors including aging and oxidative stress.^{17,18} Here, we show that Hsp26 is also required for the development of tolerance to ethanol, although mutations in the gene do not affect the response of *Drosophila* to the sedating effects of ethanol. Interestingly, the ethanol tolerance phenotype of *hsp26* is sensitive to the level of the gene product; however, there is a threshold above which increased levels of Hsp26 have no further effect on tolerance. Hsp26 is required in neurons for normal ethanol-induced behaviour.

Sedation assay measures rapid tolerance in *Drosophila*

We developed an assay to measure tolerance to the sedative effects of ethanol in *Drosophila*. Using this assay, rapid tolerance, which is induced by exposure of flies to a single moderately high ethanol dose, was quantified as a change in sensitivity observed upon administration of a second dose of ethanol after the first dose was completely metabolised.³⁰ The quantitative aspects of ethanol tolerance in flies, such as the extent of maximal tolerance and the kinetics of its decay (dissipation) were similar to those described

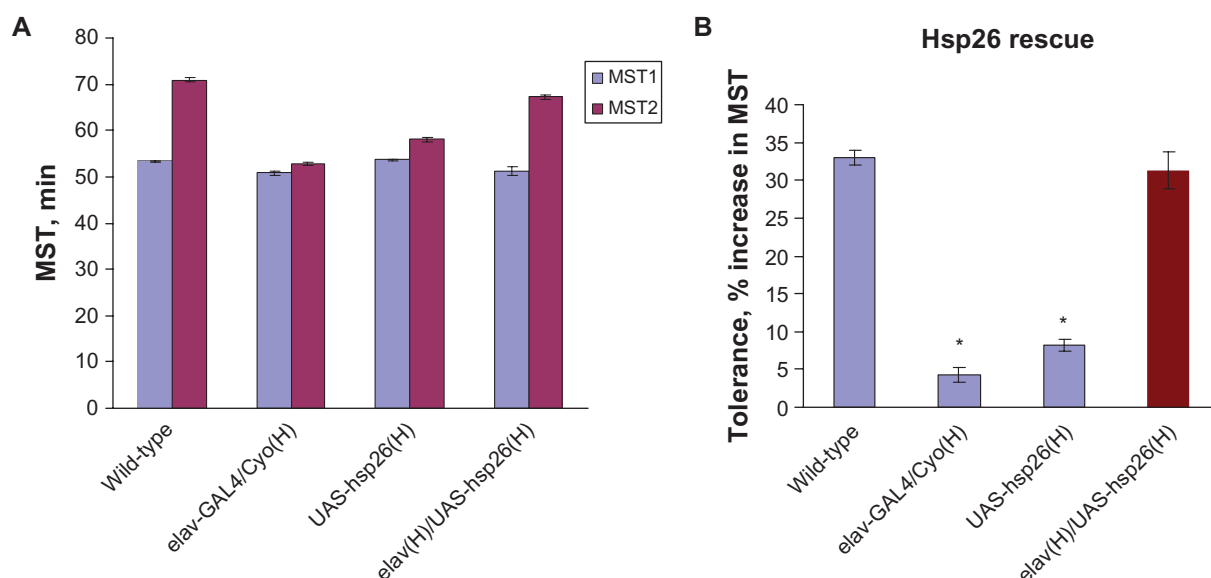


Figure 6. The *hsp26*^{EY10556} tolerance defect can be rescued by expression of a UAS-*hsp26* transgene in the nervous system under the control of the *elav*-GAL4 driver. **A**) Measures of 1st (blue bars) and 2nd (purple bars) exposures of flies in the sedation assay. **B**) Mutant flies in an *hsp26*^{EY10556} background (denoted with H in figure) carrying either transgene alone show a marked defect in tolerance development similar to that of *hsp26*^{EY10556} mutant flies, whereas flies carrying both transgenes show normal tolerance.

Notes: * $P < 0.0001$; $n = 5$ experiments. In all panels, error bars represent s.e.m.

using the inebriometer.⁵ In addition, it was shown that octopaminergic systems previously implicated in a component of rapid tolerance⁵ were also involved in impaired ethanol tolerance in this assay. Taken together, these data demonstrate that the sedation assay is a suitable paradigm in which to measure the tolerance of *Drosophila* to the sedative effects of ethanol.

Hsp26 ethanol tolerance and the molecular pathways involved

Scholz and colleagues⁶ have shown that the development of rapid tolerance in *Drosophila* is mediated by at least two molecular pathways. One is specific to ethanol tolerance and requires the neuromodulator, octopamine. The other pathway is a cellular stress response that is also induced by heat-shock. Using tolerance to sedation, we have also shown the requirement for the cellular stress pathway in the acquisition of rapid tolerance. Pre-exposure of flies to ethanol can be mimicked by heat-shock, resulting in a 42% increase in MST when exposed to ethanol four hours later, an increase that is reduced to only 10% in *hsp26* mutants. Mutations in the gene *hangover*, a nuclear zinc-finger protein, also affect this pathway but do not impinge on the ethanol-specific pathway. Hsp26, however, appears to be required for both of these pathways as mutations in this gene completely

abolish tolerance. Although *hsp26* mutations eliminate ethanol-induced tolerance, *hsp26* flies do have the ability to develop some heat-shock ethanol cross-tolerance. As noted above, there is a small (10%) amount of residual tolerance in *hsp26* flies after heat-shock, but not after prior exposure to ethanol. Interestingly, the maximum amount of tolerance induced after heat-shock is about 8% higher than that induced by ethanol (40.8% cf 33.0%). This suggests that the residual tolerance seen in *hsp26* flies is due to yet a third tolerance pathway that can be activated by stressors other than ethanol.

Hsp26 regulates distinct ethanol-induced behaviour in *Drosophila*

Hsp26 is not required for all ethanol-induced behaviours. Sedation is normal in *hsp26* mutants, showing that sedation and rapid tolerance are mediated, at least in part, by distinct molecular pathways. The relationship between sedation and tolerance is probably complex, however, as several genes have been identified that disrupt both sensitivity and tolerance to ethanol.^{10–13} To untangle the underlying mechanisms will require further study of the action of individual genes.

Why Hsp26 had distinct roles in sensitivity to ethanol and in the acquisition of tolerance was at first, surprising, given that it is widely expressed in *Drosophila*.

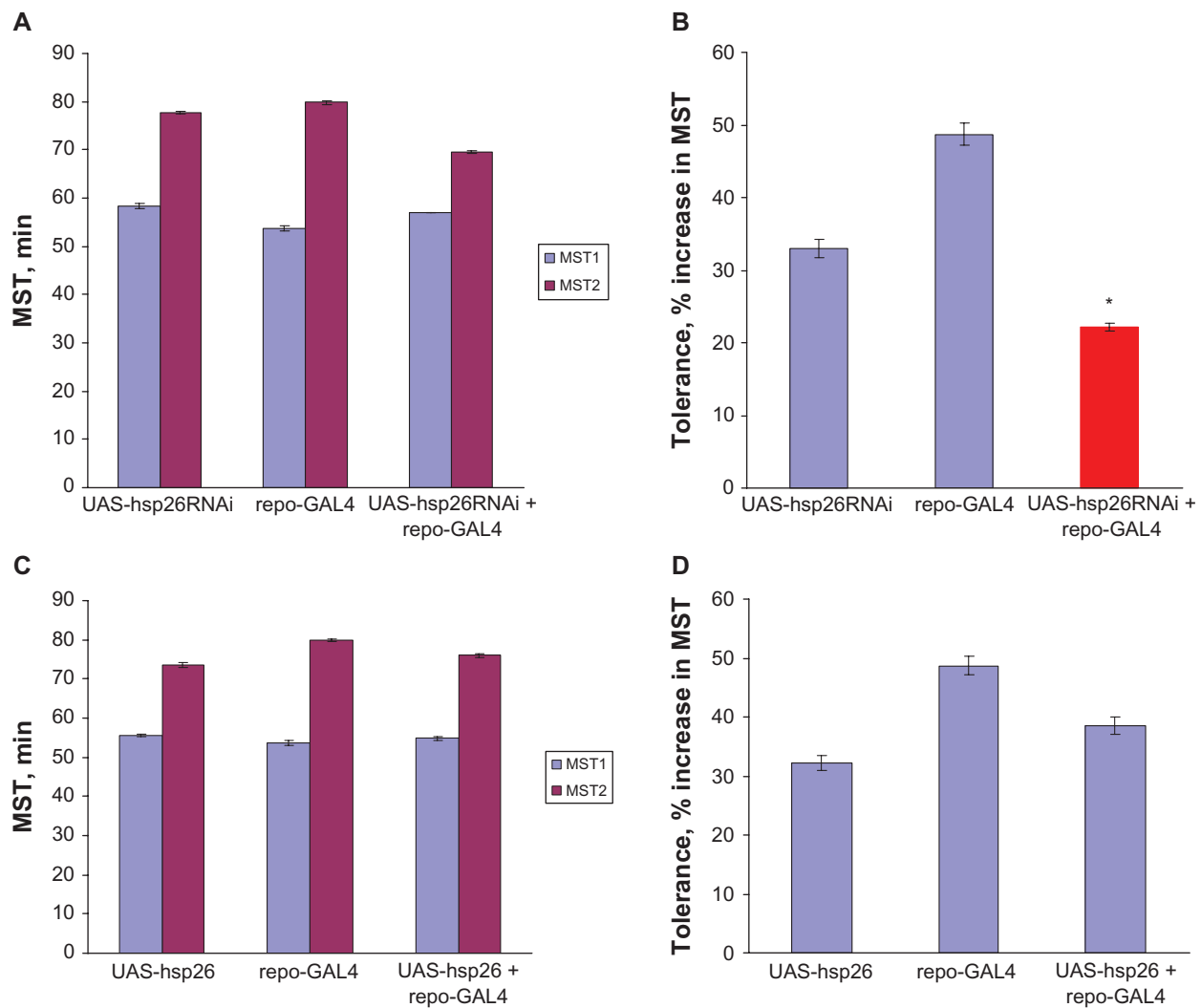


Figure 7. Effect of glia on *hsp26* ethanol tolerance. Upper and lower left panel (A, C) show the mean sedation time (MST) from the sedation assay of naïve flies (first exposure, blue bars) and flies pre-exposed to ethanol tolerance (second exposure, purple bars). Upper and lower right panel (B, D) show the development of ethanol tolerance, expressed as a percentage increase in MST between the two exposures. B) *hsp26*^{RNAi} expression when driven by repo-GAL4 shows significantly reduced ethanol tolerance compared with either transgene alone. D) Conversely, increased expression of *hsp26* in the glia did not alter ethanol tolerance.

Notes: * $P < 0.001$; $n = 5$ experiments. In all panels, error bars represent s.e.m.

However, the sHsp protein family has expanded and diversified in metazoans and it is possible that this diversification may have been driven by the acquisition of specific functions for sHsps during evolution.³¹ In *Drosophila*, for example, although both Hsp26 and Hsp27 are required for an increase in lifespan and elevated resistance to oxidative stress, only Hsp27 can partially block apoptosis.¹⁸

Hsp26 regulates ethanol tolerance by acting within the nervous system

Our present study has shown that silencing of the *hsp26* gene in the neurons reduced tolerance to ethanol while the ethanol tolerance defect seen in *hsp26* mutant

flies can be rescued by pan-neuronal expression of wild-type *hsp26*, thus supporting the hypothesis that *Drosophila* Hsp26 regulates ethanol tolerance through functions in the nervous system. This raises the possibility that Hsp26 may be acting through a mechanism to protect the neural cells from ethanol-induced damage, thus maintaining the integrity of the neurons through the promotion of neural cell stabilisation and survival. Our finding agrees with previous studies that have mapped tolerance to specific brain regions in *Drosophila* including the ellipsoid body neurons¹² and subset of neurons in the central complex.⁵ However, we also observed that a reduction in the level of Hsp26 using RNAi in glia lowered tolerance



to ethanol. Thus, the observation that wild-type levels of tolerance can be achieved by pan-neuronal expression of Hsp26 indicates that any requirement for the function of this protein in glia can be bypassed.

Hsp26 expression and behavioural response to ethanol

Hsp26 is induced in response to stress in a variety of organisms. As ethanol is a cellular stressor, it is not surprising that *hsp26* and other stress-induced proteins are highly upregulated following acute ethanol exposure in *Drosophila*.¹³ This upregulation together with our finding that Hsp26 is critical for at least one ethanol-induced behaviour has important functional consequences for the cell. In order to acquire tolerance, *Drosophila* must first be challenged by a moderate dose of ethanol. *Drosophila* cells then undergo a series of biochemical and physiological changes not only in attempt to cope with the ethanol stress but also in enhancing the survival of the stressed cells following exposure. Thus, a hypothesis to explain the role of this protein in tolerance would be that it plays a protective role in the nervous system. In addition, the upregulation of this protein following ethanol sedation may occur to ensure cellular protein homeostasis and cell protection from ethanol insults.

In conclusion, further study of the role of *hsp26* in neuronal adaptation to ethanol together with identification of its downstream targets could provide a useful hint for understanding the development of tolerance and dependence in alcoholics. Nevertheless, this study has established that *Drosophila hsp26* plays a crucial role in ethanol tolerance.

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Disclosure

This manuscript has been read and approved by all authors. This paper is unique and is not under consideration by any other publication and has not been published elsewhere. The authors and peer reviewers of this paper report no conflicts of interest. The authors confirm that they have permission to reproduce any copyrighted material.

References

1. Fadda F, Rossetti ZL. Chronic ethanol consumption: from neuro-adaptation to neurodegeneration. *Prog Neurobiol*. 1998;56:385–431.
2. Tabakoff B, Cornell N, Hoffman PL. Alcohol tolerance. *Ann Emergen Med*. 1986;15:1005–12.
3. Diamond I, Gordon AS. Cellular and molecular neuroscience of alcoholism. *Physiol Rev*. 1997;77:1–20.
4. Kalant H, LeBlanc AE, Gibbins RJ. Tolerance to, and dependence on, some non-opiate psychotropic drugs. *Pharmacol Rev*. 1971;23:135–91.
5. Scholz H, Ramond J, Singh CM, Heberlein U. Functional ethanol tolerance in *Drosophila*. *Neuron*. 2000;28:261–71.
6. Scholz H, Franz M, Heberlein U. The *hangover* gene defines a stress pathway required for ethanol tolerance development. *Nature*. 2005;436:845–7.
7. Cowmeadow RB, Krishnan HR, Atkinson NS. *Slowpoke* gene is necessary for rapid ethanol tolerance in *Drosophila*. *Alcohol Clin Exp Res*. 2005;29:1777–86.
8. Cowmeadow RB, Krishnan HR, Ghezzi A, Al'Hasan YM, Wang YZ, Atkinson NS. Ethanol tolerance caused by *slowpoke* induction in *Drosophila*. *Alcohol Clin Exp Res*. 2006;30:745–53.
9. Li C, Zhao X, Cao X, Chu D, Chen J, Zhou J. The *Drosophila* homolog of *jwa* is required for ethanol tolerance. *Alcohol and Alcohol*. 2008;43:529–36.
10. Berger KH, Kong EC, Dubnau J, Tully T, Moore MS, Heberlein U. Ethanol sensitivity and tolerance in long-term memory mutants of *Drosophila melanogaster*. *Alcohol Clin Exp Res*. 2008;32:895–908.
11. Morozova TV, Anholt RR, Mackay TF. Transcriptional response to alcohol exposure in *Drosophila melanogaster*. *Genome Biology*. 2006;7:R95.
12. Urizar NL, Yang Z, Edenberg HJ, Davis RL. *Drosophila Homer* is required in a small set of neurons including the ellipsoid body for normal ethanol sensitivity and tolerance. *J Neurosci*. 2007;27:4541–51.
13. Kong EC, Allouche L, Chapot PA, et al. Ethanol-regulated genes that contribute to ethanol sensitivity and rapid tolerance in *Drosophila*. *Alcohol Clin Exp Res*. 2010;34:302–16.
14. Hartl FU, Hayer-Hartl M. Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science*. 2002;295:1852–8.
15. Haslbeck M, Walke S, Stromer T, et al. Hsp26: a temperature-regulated chaperone. *EMBO J*. 1999;18:6744–51.
16. Jakob U, Gaestel M, Engel K, Buchner J. Small heat shock proteins are molecular chaperones. *J Biol Chem*. 1993;268:1517–20.
17. Wang HD, Kazemi-Esfarjani P, Benzer S. Multiple-stress analysis for isolation of *Drosophila* longevity genes. *Proc Natl Acad Sci U S A*. 2004;101:12610–5.
18. Liao PC, Lin HY, Yuh CH, Yu LK, Wang HD. The effect of neuronal expression of heat shock proteins 26 and 27 on lifespan, neurodegeneration, and apoptosis in *Drosophila*. *Biochem Biophys Res Commun*. 2008;376:637–41.
19. Marin R, Valet JP, Tanguay RM. *hsp23* and *hsp26* exhibit distinct spatial and temporal patterns of constitutive expression in *Drosophila* adults. *Dev Genet*. 1993;14:69–77.
20. Sirotkin K, Davidson N. Developmentally regulated transcription from *Drosophila melanogaster* chromosomal site 67B. *Dev Biol*. 1982;89:196–210.
21. Claridge-Chang A, Wijnen H, Naef F, Boothroyd C, Rajewsky N, Young MW. Circadian regulation of gene expression systems in the *Drosophila* Head. *Neuron*. 2001;32:657–71.
22. Sharma P, Asztalos Z, Ayyub C, et al. Isogenic autosomes to be applied in optimal screening for novel mutants with viable phenotypes in *Drosophila melanogaster*. *J Neurogenet*. 2005;19:57–85.
23. Sepp KJ, Schulte J, Auld VJ. Peripheral glia direct axon guidance across the CNS/PNS transition zone. *Dev Biol*. 2001;238:47–63.
24. Wen T, Parrish CA, Xu D, Wu Q, Shen P. *Drosophila neuropeptide F* and its receptor, NPFR1, define a signaling pathway that acutely modulates alcohol sensitivity. *Proc Natl Acad Sci U S A*. 2005;102:2141–6.
25. Moore MS, DeZazzo J, Luk AY, Tully T, Singh CM, Heberlein U. Ethanol intoxication in *Drosophila*: genetic and pharmacological evidence for regulation by the cAMP signaling pathway. *Cell*. 1998;93:997–1007.
26. Alexandre H, Ansanay-Galeote V, Dequin S, Blondin B. Global gene expression during short-term ethanol stress in *Saccharomyces cerevisiae*. *FEBS Lett*. 2001;498:98–103.



27. Treweek TM, Lindner RA, Mariani M, Carver JA. The small heat-shock chaperone protein, [alpha]-crystallin, does not recognise stable molten globule states of cytosolic proteins. *Biochimica et Biophysica Acta (BBA)—Protein Structure and Molecular Enzymology*. 2000;1481:175–88.
28. National Institute on Alcohol Abuse and Alcoholism (NIAAA). Understanding Alcohols: Investigation Biology and Behaviour. NIH Curriculum Supplement Series Grades. 2003:7–8.
29. Brand AH, Perrimon N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development*. 1993;118:401–15.
30. Crabbe JC, Rigter H, Uijlen J, Strijbos C. Rapid development of tolerance to the hypothermic effect of ethanol in mice. *J Pharmacol Exp Ther*. 1979; 208:128–33.
31. Haslbeck M, Miess A, Stromer T, Walter S, Buchner J. Disassembling protein aggregates in the yeast cytosol: The cooperation of Hsp26 with SSA1 and Hsp104. *J Biol Chem*. 2005;280:23861–8.

Supplementary Figure

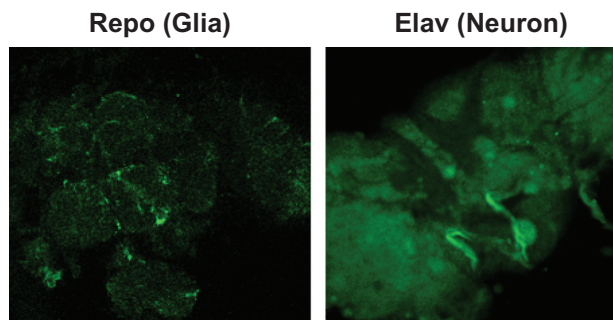


Figure S1. Confocal images of the adult *Drosophila* brain showing P[GAL4] directed expression of GFP in the brain neurons (elav-GAL4) and the glia cells (repo-GAL4).

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