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The *hangover* gene defines a stress pathway required for ethanol tolerance development

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Abstract

Repeated alcohol consumption leads to the development of tolerance, simply defined as an acquired resistance to the physiological and behavioral effects of the drug. This tolerance allows increased alcohol consumption, which over time leads to physical dependence and possibly addiction[1–3]. Previous studies showed that *Drosophila* develop ethanol tolerance with kinetics of acquisition and dissipation that mimic those seen in mammals. This tolerance requires the catecholamine octopamine, the functional analog of mammalian noradrenaline[4]. Here we describe a novel gene, *hangover*, required for normal development of ethanol tolerance. *hangover* flies are also defective in responses to environmental stressors, such as heat and the free radical-generating agent paraquat. Using genetic epistasis tests we show that ethanol tolerance in *Drosophila* relies on two distinct molecular pathways, a cellular stress pathway defined by *hangover* and a parallel pathway requiring octopamine. *hangover* encodes a large nuclear zinc-finger protein suggesting a role in nucleic acid binding. There is growing recognition that stress, at the cellular and systemic levels, contributes to drug- and addiction-related behaviors in mammals. Our studies suggest that this role may be conserved in evolution.

When flies are exposed to ethanol vapor they become hyperactive, uncoordinated, and eventually sedated. These effects of ethanol cause loss of postural control, which can be readily quantified in the inebriometer[5]. Naïve wild-type flies emerge from the inebriometer with a mean elution time (MET) of ~20 minutes at standard ethanol vapor concentrations[6,7]. A single exposure to ethanol in the inebriometer leads to the development of tolerance; flies reintroduced into the apparatus 4 hours after their initial exposure elute with a MET of ~28 minutes[4] (Fig. 1a). This acquired resistance, or tolerance, correlates with an increase in the absorbed ethanol levels needed to induce loss of postural control, and is measured as the % increase (~35–40% at standard ethanol concentrations) in MET between the first and second ethanol exposures[4].

To identify molecules and pathways involved in tolerance development, we carried out a screen for P element-induced mutants with aberrant tolerance (see Methods). Because the degree of tolerance is proportional to the length of initial ethanol exposure[4], we limited our screen to strains that reacted normally to their first ethanol exposure. One mutant strain, AE10, that showed a normal initial MET, but a reduced ability to develop tolerance (14±3% compared to 35±2% for controls), was named *hangover* (*hang*)(Fig. 1a). The tolerance defect in $hang^{AE10}$ is not simply due to a change in the rate of tolerance acquisition, as the mutant flies were also impaired when tested in a paradigm that induces maximal tolerance, through several consecutive ethanol exposures[4] (Fig. 1b). *hang* flies show normal ethanol absorption and

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metabolism (Suppl. Fig. 1a); their phenotype is therefore not due to altered drug pharmacokinetics.

The P-element in *hang*^{AE10} is inserted in the predicted open reading frame of a novel gene, CG32575 (http://www.fruitfly.org/; Fig. 2a), encoding a protein with 15 nucleic acid-binding zinc-finger domains, two of which are of the U1 subclass found in RNA-binding proteins[8] (Fig. 2b). The P-element insertion causes the mutant phenotype, as precise excision of the transposon causes reversion to the wild-type phenotype (Suppl. Fig. 1b). *hang* is expressed ubiquitously in the nervous system (Suppl. Fig. 2a,b) and HANG protein is localized to the nuclei of neurons (Fig.2c and Suppl. Fig. 2c). *hang*^{AE10} appears to be a null allele, as the ~ 7 kb mRNA encoded by *hang* (Fig. 2d) and the HANG protein (Fig. 2c) are undetectable in the mutant. Consistent with a role for *hang* in the nervous system, expression of a *UAS-hang* transgene under the control of the pan-neuronal *Appl-GAL4* driver[9] restores normal ethanol, tolerance to *hang*^{AE10} flies (Fig. 1c).

Ethanol, at high concentrations, is known to induce cellular responses similar to those elicited by heat shock[10,11]. Indeed, ethanol exposure in adult flies induces the expression of the heat shock protein Hsp70 (data not shown). To assess if these cellular stress responses may mediate ethanol tolerance development, we asked if a heat pulse could mimic the effects of ethanol pre-exposure. Heat exposure of control flies (37°C for 30 minutes) led to a 46±4% increase in MET (compared to untreated flies) when measured in the inebriometer 4 hours later. This heat-ethanol cross-tolerance is substantially reduced in *hang*^{AE10} flies (26±4%, Fig. 3a). The fact that *hang*^{AE10} flies are deficient in both forms of tolerance leads us to conclude that the cellular changes induced by ethanol and heat overlap. However, *hang*^{AE10} flies retain some ability to develop tolerance (Fig. 1a, Fig. 3a,b), suggesting that other pathways are also involved.

We had found previously that flies lacking the neuromodulator octopamine, due to a mutation in the gene encoding Tyramine β Hydroxylase (*Tbh*)[12], show a reduction in ethanol tolerance similar to that seen with *hang*^{AE10} flies[4] (22±5% compared to 34±7% for control flies, Fig. 3b). To test if the residual tolerance seen with *hang*^{AE10} mutant flies may be mediated by octopaminergic systems (and *vice versa*), we tested flies lacking both octopamine and the *hang* gene product. We find that ethanol tolerance is almost completely abolished in *hang*^{AE10},*Tbh* double mutant flies (6±2% compared to 34±7% for control flies, Fig. 3b). Because both mutations cause complete loss of gene function (ref. 12 and Fig. 2), these data suggest that the development of ethanol tolerance relies on two parallel molecular pathways, one involving octopaminergic systems and the other the *hang* gene. The octopamine pathway is specific to ethanol tolerance, as *Tbh* flies develop normal heat-ethanol cross-tolerance (Fig. 3a). The *hang* pathway, on the other hand, involves cellular stress responses that are shared with those induced by heat. Finally, the fact that some heat-ethanol cross-tolerance remains in *hang* and *Tbh,hang* double mutant flies (Fig. 3b) suggests that heat treatment engages a third unidentified pathway that can induce ethanol resistance.

To test if *hang*^{AE10} flies are more generally deficient in their ability to deal with environmental stressors, we exposed the mutant flies to paraquat, an agent that induces the formation of reactive oxygen species (ROS) and that has been used to test for oxidative-stress responses[13]. When fed paraquat-containing food, *hang*^{AE10} flies show a dose- and time-dependent reduction in survival rate as compared to control flies (Fig. 3c, d). This reduced survival was not seen with *Tbh* flies, which appear to be even slightly resistant to the effects of the drug (Fig. 3c, d). The generation of endogenous ROS is believed to contribute to organismal aging[14]. We find that *hang*^{AE10}, but not *Tbh* flies, show a reduced life span (Suppl. Fig. 3a, b), which may be caused by an impaired ability to cope with ROS. Mutations that cause neurodegeneration often show reduced lifespan[15]. This does not appear to be the case in *hang*^{AE10} flies, as their overall brain structure is normal, even in old flies (Suppl. Fig. 3c). This

suggests a more direct involvement of *hang* in the functional response to environmental stressors. *hang* expression was not altered by ethanol exposure (data not shown). However, the predicted hang protein contains, in addition to multiple zinc-fingers, a calcium-binding EF hand (Fig. 2b), suggesting that hang function may be regulated by calcium. As calcium levels are sensitive to ethanol, ROS, and heat exposure[10,16–18], this provides a potential mechanism by which these environmental stressors may regulate HANG function.

In summary, development of ethanol tolerance in *Drosophila* engages two systems that function in parallel, one involving a cellular stress pathway defined by the *hang* gene and the other involving octopaminergic systems. Octopamine has recently been implicated in the formation of appetitive (sugar-reinforced) memories in *Drosophila*[19]. The contribution of learned behaviors and stress, at the cellular and systemic levels, to drug- and addiction-related behaviors in mammals is increasingly being recognized[20,21]. Our studies in *Drosophila* suggest that these pathways are conserved allowing their analysis in this genetically tractable model organism.

Methods

Fly stocks and genetics

We generated and tested 404 homozygous viable X-linked lines carrying PZ(ry^+)-element insertions[22] for their ability to develop ethanol tolerance. Lines were generated[6] and tested as described before[4]. Two lines with altered ethanol tolerance were isolated, one of them the insertion line $hang^{AE10}$. To insure that the phenotype was not caused by chromosomal alterations unlinked to the PZ(ry^+)-element, $hang^{AE10}$ mutants were crossed for 5 generations to ry^{506} flies and re-tested for tolerance; $hang^{AE10}$ flies retained their ethanol tolerance defect. The *PZ(+)* control line displays normal ethanol sensitivity and tolerance as measured in the inebriometer[6,4]. Excisions of the $hang^{AE10}$ PZ(ry^+)-insertion were generated as described before[6]. Four independent excision stocks were tested for ethanol tolerance and analyzed by PCR and DNA sequencing; a perfect correlation between precise excision of the P element and phenotype reversion was observed (Suppl. Fig. 1b).

The $T{\square}h^{nM18}$,$hang^{AE10}$double mutant stock was generated by recombination as both genes reside on the X chromosome. Stocks that displayed female sterility, associated with the $T{\square}h^{nM18}$ mutation [12], were tested for β-galatosidase expression to determine the presence of the $hang^{AE10}$ PZ(ry^+)-insertion. A fertile ($T{\square}H^+$) w^{1118}, $hang^{AE10}$ strain was also generated as a control. Two independent recombinant strains (w^{1118}, $T{\square}h^{nM18}$, $hang^{AE10}$) were tested for ethanol tolerance with identical results.

Behavioral analyses

For all behavioral tests flies were generated as described before[6,4]. Approximately 100 3-4 day old males were tested in the inebriometer (MET1)[6,7]. After the initial exposure, flies were collected in vials and allowed to recovered in a humidified incubator at 25°C. The second exposure in the inebriometer (MET2) was initiated exactly 4hrs after the start of the first exposure. Tolerance was calculated as described before[4] (MET2- MET^1/MET^1 x 100). Ethanol absorption was quantified as described before in extracts prepared from adult flies[6,7]. Significance was established using either Student's paired *t*-tests assuming equal variance or one-way ANOVAs with Newman-Keuls post hoc tests.

For heat shock-ethanol cross-tolerance experiments, flies were incubated in a vial at 37°C for 30 min in a water bath. After a recovery period of 3 hrs and 30 min at 25°C in a humidified incubator, these flies were exposed to ethanol in the inebriometer (MET^{hs+}). Tolerance was

calculated with respect to MET^{hs-}, the MET of flies that were not heat-treated (MET^{hs+}-MET^{hs-}/MET^{hs-} x 100).

To measure sensitivity to paraquat groups of 20, 3-4 day old male flies were transferred into a glass vial containing a filter paper saturated with aqueous solutions of paraquat (0, 1, 5, 10 and 20 mM) in 5% sucrose. Flies were kept in an incubator with 70% humidity at 25°C. Every 12 hrs dead flies were counted and live flies were transferred into new vials.

Molecular Biology

The genomic DNA flanking the *hang*^{AE10} PZ(ry^+) insertion was isolated by plasmid rescue. Comparisons with the published genome sequence of *Drosophila* revealed that the insertion was located in the first exon of CG32575, a finding that was confirmed by PCR. Several cDNA clones (GH14331, LD34334 and GH19829) were sequenced. The GH14331 cDNA is approximately 7kb long and its sequence corresponds to the predicted structure of CG32575 (http://www.fruitfly.org/). The sequence of GH14331 does not differ from the genomic sequence, but differs from annotated transcripts CG32575RA and RB. The full-length cDNA of GH14331 was cloned into pUAST[23] to generate the *UAS-hang* transgene. For Northern blots, 1μg $polyA^+$ RNA from *PZ(+)* and *hang*^{AE10} was used. A 3.7 Kb EcoRI/KpnI fragment from the GH14331 cDNA clone, spanning from exon 1 (3' of the insertion site) to exon 5, was used as a hybridization probe. A probe that recognizes *Tubulin 84B* mRNA (nucleotides 10–340; http://flybase.bio.indiana.edu/) was used as a control.

Histology

To generate antibodies to the HANG protein, a His-tagged antigen was made. The sequence coding for amino acids 62-319 of HANG were cloned into the pET28-b vector (Novagene). Protein was purified from E. coli extracts using a nickel column as indicated by manufacturers, and injected into rabbits. For HANG immunohistochemistry, flies were fixed for 2.5 hrs in 5% paraformaldehyde at 4°C, washed and infiltrated with a 25% sucrose solution overnight at 4° C. Whole flies were sectioned into 10 μm slices using a cryostat. Sections were washed twice for 5 min in PBT (PBS plus 0.3% Triton X-100) and preincubated with 10% normal goat serum in PBT for 2 hrs at room temperature. Incubation with anti-HANG serum (1:5000 in PBT plus 10% NGS) was carried out overnight at 4°C. After two 5-minute washes with PBT, signal detection was carried out using the Vectastain® ABC elite kit with a peroxidase-coupled mouse IgG antibody.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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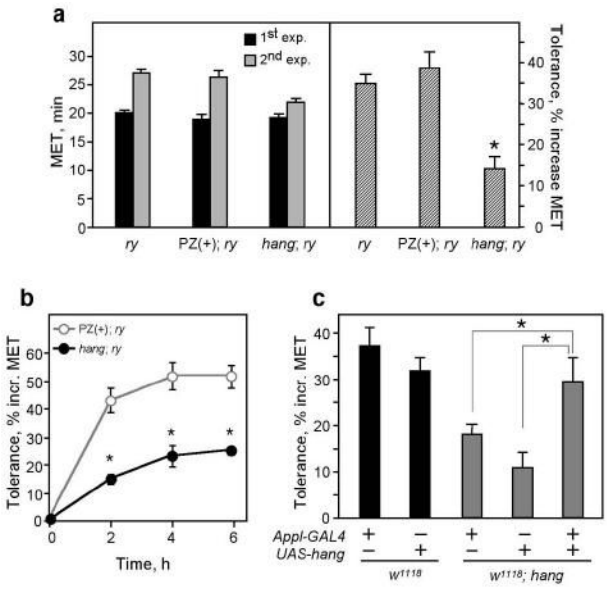

Figure 1.

hang^{AE10} is an ethanol tolerance mutant.

a. Left panel: Mean elution times (METs) from the inebriometer of naïve (black bars) and ethanol pre-exposed (gray bars) flies. Right panel: Tolerance development, expressed as a % increase in MET between the two exposures. *hang*^{AE10} mutants (*hang;ry*) show significantly reduced tolerance compared to two controls, (*ry*) and (*PZ(+);ry*). n=18 experiments, *p<0.0001. In all figures, error bars correspond to the standard error of the mean (SEM).

b. *hang*^{AE10} flies are defective in a chronic tolerance paradigm, in which flies are exposed to ethanol in the inebriometer four times at 2-hour intervals. n=8 experiments, *p<0.001.

c. The *hang*^{AE10} tolerance defect can be rescued by expression of a *UAS-hang* transgene in the nervous system under the control of *Appl-GAL4*. Mutant *w*^{1118}*,hang* flies carrying either transgene alone show reduced tolerance similar to that of *hang*^{AE10} flies, while flies carrying both transgenes show normal tolerance. n=5–10 experiments, *p<0.005.

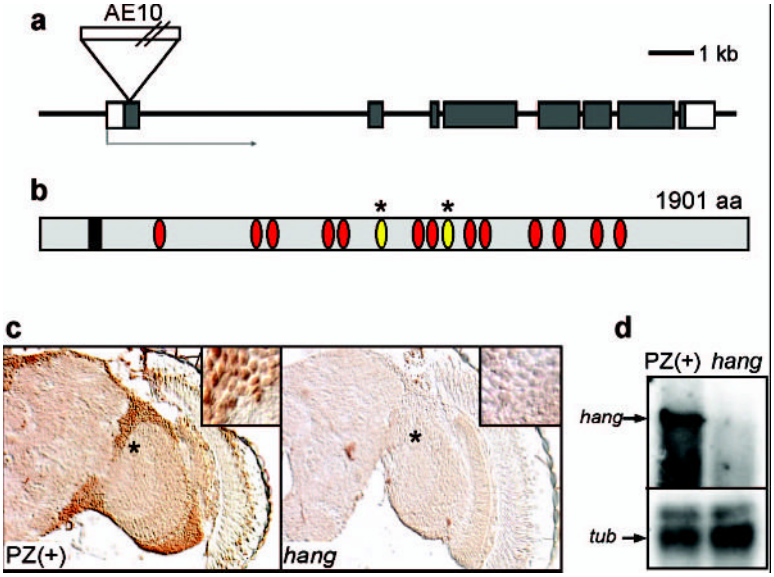

Figure 2.

The *hang*^{AE10} mutation disrupts a gene encoding a novel zinc-finger protein. a. Genomic map of the *hang* locus (CG32575). The 8 exons are shown as boxes; gray shading indicates protein-coding regions. The position of the P-element insertion AE-10 is shown.

b. Conceptual translation predicts a protein of 1901 amino acids (http://www.fruitfly.org/). The positions of an EF hand (box) and zinc-finger domains (ovals) are indicated; the U1-like zinc-fingers are marked with asterisks.

c. HANG protein is expressed broadly in the nervous system (left panel) and localized to nuclei of neurons (see also Suppl. Fig. 2). Expression is undetectable in the *hang*^{AE10} mutant (right panel).

d. Northern blot analysis shows that expression of the full length *hang* transcript (~7Kb in length, upper arrow) is absent (or severely reduced) in the *hang*^{AE10} mutant compared to the *PZ(+)* control strain. The lower panel (*tub*) shows expression of *Tubulin 84B*, used as a control.

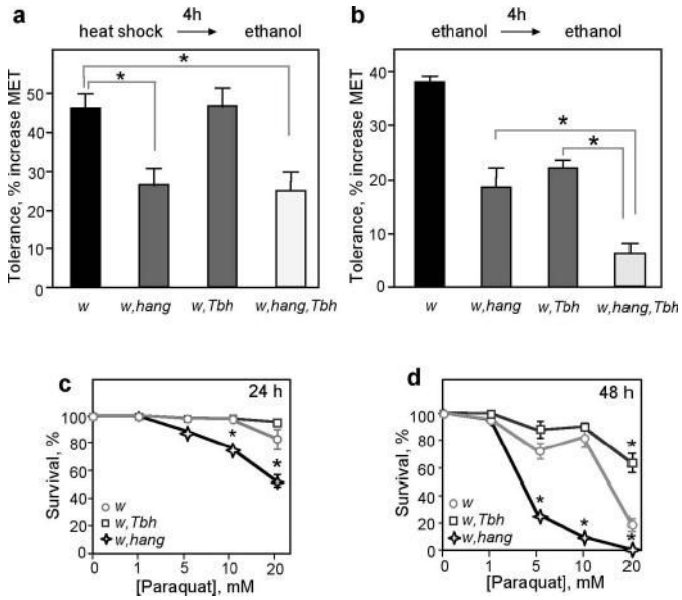

Figure 3.

hang^{AE10} shows impaired heat-ethanol cross-tolerance. a. Heat-ethanol cross-tolerance: Control flies (*w*) and flies lacking octopamine (*w,T□h*) show normal cross-tolerance, while *hang*^{AE10} mutants (*w,hang*) and the double mutant (*w,T□h,hang*) display strongly reduced cross-tolerance. n=8–10 experiments, *p<0.0001.

b. Ethanol tolerance, while reduced in *hang*^{AE10} and *T□h* flies (significant difference with control not indicated in panel), is essentially absent in the *T□h,hang* double mutant flies. n=4–6 experiments, *p<0.001.

c, d. *hang*^{AE10} flies show increased sensitivity to paraquat. Panel c and d depict survival data obtained after 24 and 48 hours, respectively. n=5 experiments, *p<0.001.