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Stress Response Genes Affect Ethanol Tolerance in *Drosophila*

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Abstract
The role of stress pathway in the behavioural responses to ethanol has been previously documented; however, the molecular mechanism governing tolerance of stress genes to ethanol sedation is yet to be fully elucidated. To study the interaction between stress response genes and ethanol tolerance in Drosophila, this study examined individual flies carrying mutations in the highwire (hiw^{ND8}, hiw^{EP1305} and hiw^{EP1308}), heat shock protein 83 ($Hsp83^{08445}$ and $Hsp83^{e6A}$) and multi-protein bridging factor 1 ($mbf1^{2}$) genes and showed that after 4 h of initial ethanol sedation, both the mbf1 and the Hsp83 mutants exhibited a substantial reduction in ethanol tolerance compared to their control flies. Conversely, two of the three hiw alleles displayed a sex-specific increase in ethanol tolerance compared to their control flies. All of these stress response genes are evolutionarily conserved and their roles in ethanol tolerance which is a precursor to alcohol addiction may help shed light on the biology underlying the complex phenomenon of alcohol dependence.

Keywords: hiw, mbf1, hsp83, ethanol, stress, tolerance, sedation.

Introduction

Alcohol intake in humans causes long-term physiological changes including tolerance, which in turn encourages increased alcohol consumption, development of physical dependence and addiction (Tabakoff *et al*., 1986). In addition, studies have shown that tolerance to alcohol, which is regarded as an addictive drug; is influenced genetically. This genetic disposition to alcohol tolerance may be a good predictor for alcohol addiction and dependence (Awofala, 2013). The transition to alcohol dependence involves the impairment of not only the neuronal circuits involved in reward system, but also the circuits that mediate behavioural responses to stressors (Gilpin & Koob, 2008). Ethanol alters the expression of many genes, including stress proteins and chaperones, through its interaction with many targets that produce second messengers and also through interaction with ion channels, transporters, neurotransmitter receptors and enzymes (Diamond & Gordon, 1997). The fruit fly, *Drosophila melanogaster,* is a genetic workhorse for studying genes and molecules underlying the behavioural tolerance to ethanol sedation (Moore *et al*., 1998; Awofala, 2012b; Awofala *et al*., 2011). Studies employing *Drosophila*, have indicated that stress response may mediate the development of ethanol tolerance (Scholz *et al.,* 2000; Scholz *et al.*, 2005; Awofala *et al.*, 2011). For instance, a transcription factor known as *hangover* encoding a zinc finger protein has been shown to be deficient in ethanol tolerance. The same transcription factor *hangover*, has also been shown to be deficient in heat-ethanol cross-tolerance, indicating that cellular changes induced by ethanol and heat overlap (Scholz *et al.*, 2005). In addition, many genes related to stress responses, including approximately half of all *Drosophila* heat shock protein genes, and genes involved in the regulation of oxidative stress and aging have been shown to be upregulated after ethanol exposure in transcriptional profiling studies (Morozova *et al.*, 2006, Kong *et al*., 2010, Awofala, 2011b; Awofala *et al.*, 2012). Interestingly, a member of the heat shock protein genes, heat shock protein 26 gene (which is reportedly upregulated by ethanol exposure in these transcriptional profiling studies), has been shown to be required for ethanol tolerance in *Drosophila* (Awofala *et al*., 2011).

Other stress-related genes in *Drosophila* with recent roles in ethanol sedation include the neuronal protein *highwire* (*hiw*) gene, the transcriptional regulator *multi-protein bridging factor 1* (*mbf1*) gene and the *heat shock protein 8*3 gene (*hsp83*). Notably, while both the *hiw* and the *mbf1* genes were implicated in increased sensitivity to the sedating effect of ethanol, flies carrying mutations in the *hsp83* gene displayed reduced sensitivity to the sedating effects of ethanol (Awofala *et al.*, 2012). Given the known role of stress pathways in the development of alcohol addiction, this study hypothesises that these stress genes may be involved in the development of ethanol tolerance, thereby making them a focus of this study.

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Materials and Methods

Source and maintenance of flies

Flies were cultured at 18°C in an equal 12 h light and dark cycle on standard maize meal food sprinkled with a small amount of dried baker's yeast as described by Awofala (2010). w^{+}*; Iso2C; Iso 3I* (control flies designated *Ctl*) isogenised on the second and third chromosome was obtained from Cahir O' Kane (University of Cambridge), hiw^{ND8} from A. DiAntonio (Washington University in St. Louis), $mbf1^{2}$ and the control $P[mbf1^{+}]; mbf1^{2}$ flies from S. Hirose (National Institute of Genetics, Mishima, Japan), and $Hsp83^{08445}$, $Hsp83^{e6A}$ (recessive lethal), hiw^{EP1305}, hiw^{EP1308} flies from the Bloomington *Drosophila* Stock Centre. The following generated 'control' stocks were also used for assessing the effects of genetic background on flies' ethanol tolerance: $Hsp83^{08445}/TM3$ (generated from a cross between male and female $Hsp83^{08445}$ with the *TM3* balancer chromosome) and *TM6B/+* (generated from a cross between $Hsp83^{e6A}/TM6B$ and the *Ctl*). 2- to 5-day-old male or female flies were used for behavioural testing.

Tolerance assay

The tolerance assay used in this study has been previously described by Awofala *et al*. (2011). 20 active and well fed males (or females depending on their suitability for behavioural testing) were selected under CO_2 anaesthesia and allowed to recover for 24 h before use for each trial. 1 ml ethanol solution at 50% concentration was added to a piece of folded Kimwipe tissue (11.4 x 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 Mean Sedation Time (MST) used as a measure of the resistance to the sedative effects of ethanol (Awofala, 2011a; Awofala, 2012a) was calculated as the sum of the number of flies sedated at every 6 min 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. After initial exposure, the Mean Sedation Time 1 (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 with Mean Sedation Time 2 (MST2) was initiated exactly 4 h after the start of the first exposure. Tolerance development was calculated relative to the MST of flies following their first and second exposure in the sedation paradigm.

Statistical analysis

Statistical significance was assessed by either Student's unpaired t-test assuming equal variance or one-way analysis of variance (ANOVA) with Newman-Keuls post-hoc tests.

Results

Mutations affecting the hsp83 gene showed reduced ethanol tolerance

$Hsp83^{08445}$ and $Hsp83^{e6a}$ showed decreased ethanol sensitivity (Figure 1a), they also showed marginally significantly reduced ethanol tolerance; measured 4 h after the initial ethanol exposure compared with either wild-type control (Ctl) or $hsp83^{08445}/TM3$ flies (P=0.04, Figure 1b). The $hsp83^{08445}/TM3$ flies displayed ethanol tolerance that was indistinguishable from the wild-type control when measured in the sedation paradigm. $Hsp83^{08445}$ is a viable allele of *hsp83*. However, a lethal allele $hsp83^{e6A}$, when tested over a wild-type chromosome ($hsp83^{e6A}/+$) displayed a statistically significantly reduced ethanol tolerance compared to the wild-type control (P<0.0001, Figure 1b). The reduced ethanol tolerance phenotype of $hsp83^{e6A}$ heterozygotes was significantly more severe than that of $hsp83^{08445}$ homozygotes (P<0.0001). $Hsp83^{08445}$, also known as *scratch* is a mutant for Hsp90 protein that has a P-element inserted in the 5' intron of the gene, which leads to a small reduction in Hsp90 protein (Yue et al., 1999). The $hsp83^{e6A}$ mutation on the other hand, is most likely a loss-of-function mutation or a very strong hypomorph as it is an EMS-induced mutation affecting the coding region of the exon.

Mutations affecting the mbf1 gene led to reduced ethanol tolerance

The loss of *mbf1* gene was shown to affect *Drosophila*'s sensitivity to ethanol (Figure 2a). It was also observed that $mbf1^{2}$ animals showed reduced ethanol tolerance (P<0.01) compared with $P[mbf1^{+}]$; $mbf1^{2}$ (a rescue construct containing a genomic *mbf1* in an $mbf1^{2}$ mutant background) the control strain (Figure 2b). Although, ethanol sensitivity of the control strain, $P[mbf1^{+}]; mbf1^{2}$ flies displayed substantially reduced tolerance (P<0.001) compared with wild-type flies (Ctl), (Figure 2b). $P[mbf1^{+}]; mbf1^{2}$ flies have previously been shown to rescue a number of phenotypes such as restoring the increased sensitivity of the mutant *mbf1* flies upon exposure to hydrogen peroxide to wild type level (Jindra *et al*., 2004), the behaviour displayed in the tolerance assay suggests that it is unable to rescue the tolerance phenotype to the wild-type tolerance level. A possibility is that the level and/or temporal expression of the inserted *mbf1* gene is not compatible with the acquisition of wild-type level of tolerance.

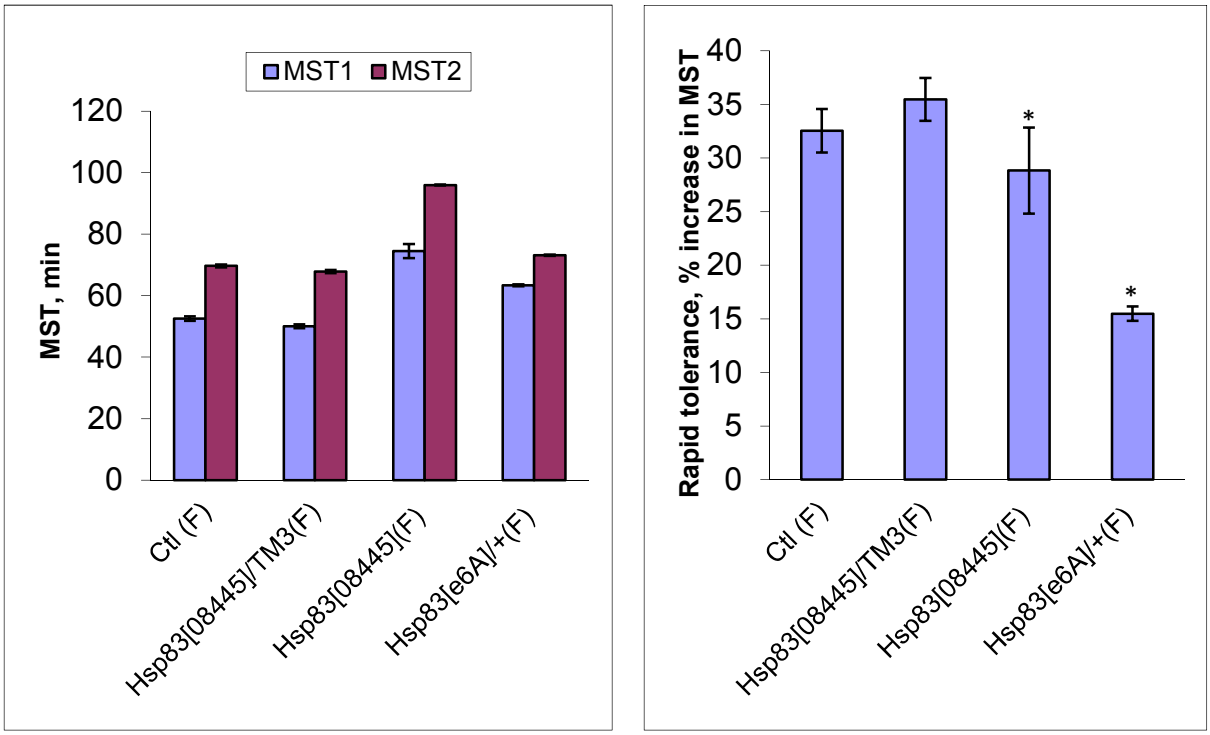

Figure 1: Effect of *hsp83* on ethanol tolerance
(a) Left panels show mean sedation time (MST) from the sedation assay of naïve flies (first exposure, first bars) and flies pre-exposed to ethanol (second exposure, second bars). (b) Right panels show development of ethanol tolerance, expressed as a percentage increase in MST between the two exposures. The two *hsp83* mutant flies displayed significantly reduced sensitivity to ethanol on first exposure in (a), they also showed a significantly reduced tolerance ($^{*}p<0.05$) compared with the wild-type controls in (b). n=6-8 and error bars represent SEM.

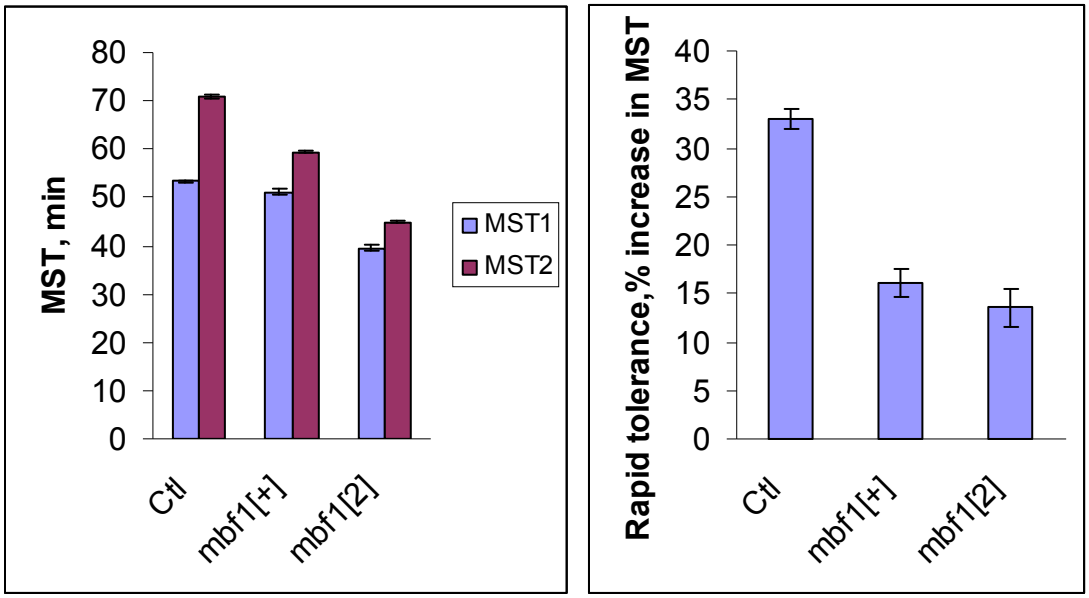

Figure 2: Effect of *mbf1* on ethanol tolerance
(a) Left panels shows mean sedation time (MST) from the sedation assay of naïve flies (first exposure, first bars) and flies pre-exposed to ethanol (second exposure, second bars). (b) Right panels shows development of ethanol tolerance, expressed as a percentage increase in MST between the two exposures. $mbf1^{2}$ mutants showed significantly reduced tolerance compared to wild-type control (Ctl), $^{*}p<0.00001$, but showed only marginally significant reduction compared to $mbf1[^{+}]$ control, $^{*}p<0.04$). n=6, error bars represent SEM.

Mutations affecting the hiw gene showed sex-specific effects on tolerance

To determine whether the *hiw* gene is implicated in ethanol tolerance, three alleles of *hiw* were tested in the sedation assay protocol. Whereas the initial ethanol sensitivity was enhanced in all the three alleles (Figure 3a), two of the alleles displayed sex-specific effects in ethanol tolerance (Figure 3b). When the male *hiw* mutants for all the three alleles were assayed for tolerance, they did not exhibit any significant alterations in ethanol tolerance; although they showed enhanced ethanol sensitivity. Conversely, two of the three female *hiw* alleles that showed enhanced ethanol sensitivity displayed statistically significant increase ($P< 0.001$) in ethanol tolerance compared with control flies (Figure 3b). The fact that these behavioural responses were seen in more than one allele of the same stocks, greatly increase the likelihood that mutations in these genes rather than unlinked second-site mutations, are responsible for the altered ethanol phenotypes. The phenotype is specific to ethanol tolerance, as mutations in both sexes have been previously reported to cause an increase in ethanol sensitivity (Awofala *et al*., 2012). The enhanced tolerance effect is highly pronounced in the hiw^{ND8} female allele with a percentage increase in tolerance of 59.1 ± 2.1 compared with 32.5 ± 2.0% for wild-type flies. The reason for this overt behaviour is not known.

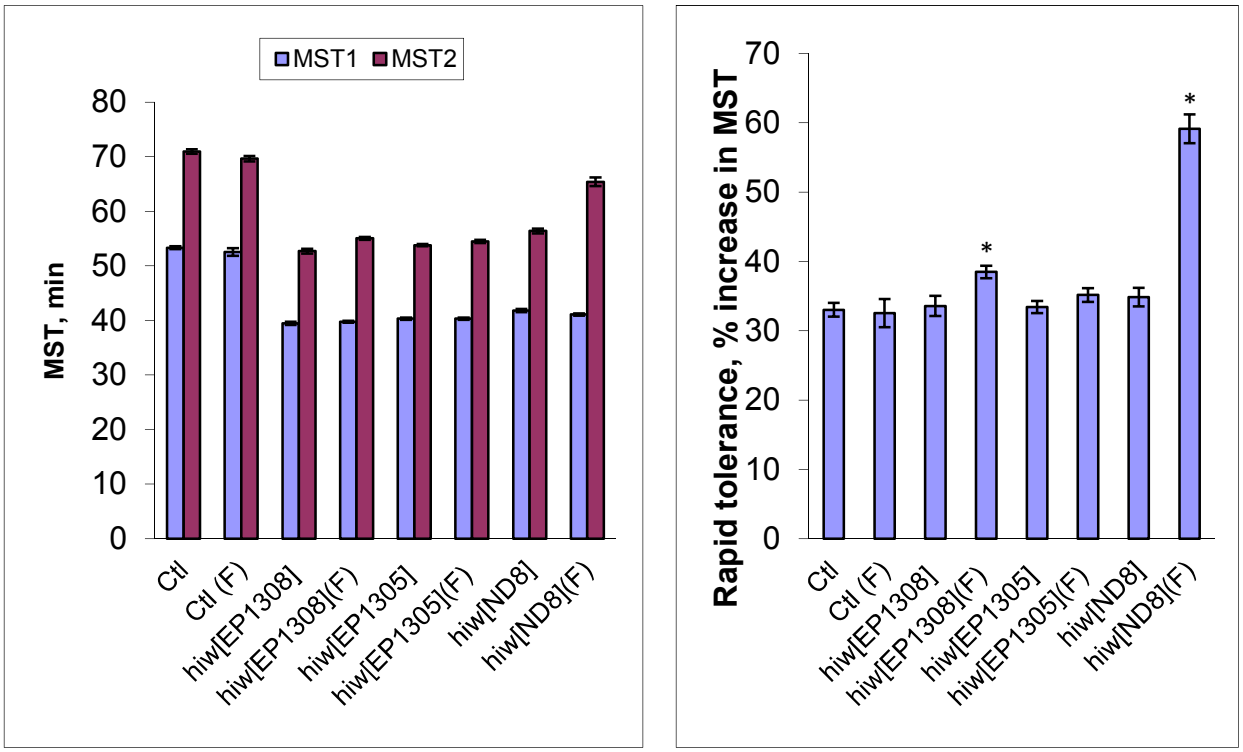

Figure 3: Effect of *hiw* on ethanol tolerance

(a) Left panels shows mean sedation time (MST) from the sedation assay of naïve flies (first exposure, first bars) and flies pre-exposed to ethanol (second exposure, second bars). (b) Right panels show development of ethanol tolerance, expressed as a percentage increase in MST between the two exposures. The male *hiw* mutants displayed non-significant ethanol tolerance from the wild-type control, while two the female *hiw* mutants of hiw^{EP1308} and hiw^{ND8} showed significantly increased tolerance ($^{*}p<0.001$) compared with the wild-type control n=5-8 and error bars represent SEM.

Discussion

To shed light on the molecular mechanism governing tolerance of stress genes to ethanol sedation, this study analysed three stress response genes (*hiw*, *hsp83*, and *mbf1*) earlier implicated in ethanol sedation sensitivity in *Drosophila* (Awofala, 2011a; Awofala *et al.*, 2012. Two mutants, $hsp83^{e6A}$ and $hsp83^{08445}$ exhibited reduced tolerance to ethanol sedation in this study. The mutant, $mbf1^{2}$ also displayed reduced tolerance to ethanol sedation. On the other hand, mutants hiw^{EP1308}, hiw^{EP1305} and hiw^{ND8} exhibited sex-specific increased ethanol tolerance.

$Hsp83^{08445}$ (known as *scratch*) was obtained in a *P* element insertional mutation screen (Karpen & Spradling, 1992; Castrillon *et al*., 1993). The *P* element in *scratch* is inserted in the intron of the *hsp83* gene located approximately 60bp from the junction of the first exon and the intron (Yue *et al*. 1999). This mutant is homozygous viable, female fertile but male sterile (Castrillon *et al*., 1993) and it is maintained over a third chromosome balancer (*TM3*).

Hsp83^{e6a} is caused by an EMS-induced mutation (point mutation) in the coding region of *hsp83* in the position of the amino acid exchanges S592F (C1775T) within the C-terminal protein domain (Yue *et al*., 1999). Hsp90 is an abundant and ubiquitous cellular protein that is indispensable for cell survival even under non-stressful conditions (Hendrick & Hertl, 1993). This protein has been shown to prevent the aggregation of chemically denatured or heat denatured proteins (Jakob *et al*., 1995). Thus, the involvement of Hsp90 in ethanol tolerance suggests that the gene (*Hsp83*^{08445}) may be an important element in regulating protein stability while protecting the cells against the depressive effects of ethanol. Interestingly, mutant flies for Hsp90 proteins mimic the behaviour displayed by *Cycle* (Cyc) loss-of-function flies by increasing their mortality rates upon sleep deprivation (Shaw *et al*., 2002). Activation of Hsp90 protein on other hand was shown to rescue *Cyc* null mutants from the lethal effects of sleep deprivation (Shaw *et al*., 2002), indicating a role of Hsp90 protein in clock homeostasis or regulation. Recently, an increase in the transcription of the gene that codes for Hsp90 protein in cultured mouse cortical neurons exposed to an acute dose of ethanol has been reported (Pignataro *et al*., 2007). Thus, the implication of Hsp90 protein in the behavioural tolerance to ethanol sedation has in itself, important implications for cellular responses to ethanol.

Drosophila highwire encodes a ubiquitin ligase protein involved in the negative regulation of synaptic growth at the *Drosophila* neuromuscular junction (NMJ). All *hiw* alleles are viable. While *hiw*^{ND8} is a loss of function mutation, *hiw*^{EP1308} and *hiw*^{EP1305} are caused by *P* element insertions in different positions within the gene (Wu *et al*., 2005). The marked interaction between sex and ethanol tolerance in *hiw* is striking. Male *hiw* flies displayed normal ethanol tolerance whereas the female flies showed enhanced ethanol tolerance to the sedative effect of ethanol. Notably, male *hsp26* flies have been shown to exhibit normal ethanol sensitivity in the sedation assay whereas female flies displayed reduced sensitivity (Awofala, 2010). Thus, the behaviour of these stress genes indicates the involvement of highly complex regulatory mechanisms in both the sensitivity and tolerance to ethanol in the two sexes. The dynamics of ethanol-induced effects are very different in the two sexes, and might shed light on the numerous of sex-specific effects found in *Drosophila* (Sørosen *et al*., 2007). Interestingly, evidence from epidemiological and clinical studies has shown notable sex differences in alcohol use and propensity for abuse and dependence (Vetter-O'Hagen *et al*., 2009). For example, differences between men and women in their sensitivity to a number of acute or chronic consequences of ethanol have been reported: adolescent males consumed more ethanol relative to their body weights than adolescent females and adults of both sexes, whereas adult females generally consumed more than adult males (Fillmore & Weafer, 2004, NIAAA, 2004; Vetter-O'Hagen *et al*., 2009). In rodents, mature females have been shown to display higher ethanol intake than their male counterparts (Lê *et al*., 2001, Chester *et al*., 2006).

Drosophila multiprotein bridging factor 1 is a transcriptional co-activator that functions to preserve redox dependent activator protein-1 (AP-1) activity during oxidative stress (Jindra *et al*., 2004). The *P* element in *mbf1*^{2} flies is inserted 21bp upstream of the first exon of the *mbf1* gene. This *mbf1*^{2} is a null allele, as the ~1.6kb transcript encoded by its protein are undetectable in the mutant as confirmed by southern blot, and western blot analyses of the mbf1 protein from adult flies (Jindra *et al*., 2004). The role of *Drosophila mbf1* has been reported to be critical when gene expression is required in response to developmental or environmental signals (Liu *et al*., 2003). *mbf1* gene is also suggested to be involved in Ca^{2+}-induced gene activation (Liu *et al*., 2003). The role of calcium in the acute action of ethanol and the development of tolerance to ethanol has also been reported (Mayer *et al*., 1980).

In summary, this study has once again demonstrated a role for stress genes in the behavioural responses to ethanol sedation, thus, reinforcing further research in this area.

Acknowledgments

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