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RESEARCH ARTICLE

Drosophila highwire gene modulates acute ethanol sensitivity in the nervous system

Awoyemi A. AWOFALA (✉)[1,2]

[1] *School of Life Sciences, University of Sussex, Falmer, Brighton BN1 9QG, UK*
[2] *Department of Biological Sciences, Tai Solarin University of Education, Ijebu-Ode, Ogun State, Nigeria*

Abstract **Animals exhibit behavioral differences in their sensitivity to ethanol, a trait that is at least in part due to genetic predispositions. This study has implicated a large neuronal protein involving Highwire, a *Drosophila* E3 ubiquitin ligase (Hiw, a homolog of Pam, a protein associated with Myc found in humans) in acute sensitivity to ethanol sedation. Flies lacking Hiw were hypersensitive to the sedating effect of ethanol whereas those overexpressing Hiw showed decreased sensitivity to ethanol. Furthermore, RNAi functional knockdown of Hiw in adult neurons or ellipsoid body neurons showed increased sensitivity to ethanol sedation. None of these manipulations of the *hiw* gene caused changes in the rate of ethanol absorption and/or metabolism. These results suggest a previously unknown role for this highly conserved gene in regulating the behavioral responses to an addictive drug.**

Keywords *hiw*, *Drosophila*, ethanol sensitivity, neurons, ellipsoid body, ubiquitin ligase

Introduction

Alterations in neuronal structure, biochemistry and function have been considered to be the driving force behind the initiation and maintenance of drug addiction and dependence (Miguel-Hidalgo, 2009). In addition, studies have shown that sensitivity to ethanol, an addictive drug, is influenced genetically and may be a good predictor for alcohol addiction and dependence (Schuckit and Gold, 1988; Schuckit et al., 1996). The neural mechanisms responsible for this initial level of response and dependence largely remain unknown. The fruit fly *Drosophila melanogaster* offers very powerful genetic tools with which to dissect conserved genes and pathways underlying this behavioral response to ethanol (Wolf and Heberlein, 2003).

Drosophila hiw encodes a large neuronal protein belonging to an evolutionally conserved family of proteins (PHR) including Phr1 in mouse, protein associated with Myc (Pam) in human, regulator of presynaptic morphology (RPM-1) in *Caenorhabditis elegans*, and Esrom in zebrafish (Han et al., 2008). Hiw consists of an N-terminal guanine-nucleotide exchange factor-like domain, two PHR repeats of unknown function, and a C-terminal RING finger (RZF) domain which is a putative E3 ubiquitin ligase domain that has been shown to be required for *hiw* function (Wu et al., 2005). Studies have demonstrated that Hiw and its homologs regulate pre-synaptic morphology (DiAntonio et al., 2001; Wan et al., 2000, 2005). Hiw restrains synaptic growth primarily by downregulation of Wallenda (Wnd), a MAPK kinase kinase (Collins et al., 2006). In addition, members of the PHR family have been suggested to mediate many biological processes including signaling pathways [e.g. BMP signaling in *Drosophila* (McCabe et al., 2004), JNK/p38 MAPK signaling in *C. elegans* (Nakata et al., 2005), and cAMP signaling pathway in mammalian cells (Pierre et al., 2004)]. The role of cAMP signaling in ethanol responses has been well documented in *Drosophila* (Moore et al., 1998), mice (Wand et al., 2001) and humans (Yamamoto et al., 2001). In addition, a role for ubiquitin in ethanol response has recently been reported: two yeast mutant strains–yeast ubiquitin ligase, Rsp5 and ubiquitin-conjugating enzyme, Ubc4 have been implicated in increased ethanol sensitivity (Hiraishi et al., 2009). Given that evolutionary conservation of functions usually exists among genes from different organisms, the possibility of similar behavioral response in *Drosophila* gene encoding an ubiquitin ligase make *hiw* a tempting focus of study.

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Correspondence: Awoyemi A. AWOFALA
E-mail: a.a.awofala@sussex.ac.uk, a.a.awofala@gmail.com

Materials and methods

Fly strains

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 behavioral tests (Sharma et al., 2005) and kindly provided by Cahir O' Kane (University of Cambridge, Cambridge, UK). Mutant strains used in this study were hiw^{EP1308}, hiw^{EP1305} from Bloomington *Drosophila* Stock Center at Indiana University (Bloomington, IN) and hiw^{ND8} flies from Aaron DiAntonio (Washington University in St. Louis). P[GAL4] lines elav; 8760 and c819 (Renn et al., 1999) were obtained from Bloomington, IN and Flytrap (http://www. flytrap.org) respectively. UAS-*hiw*, UAS-*hiw*-GFP(II), UAS-*hiw*ΔRING flies were also obtained from DiAntonio. The UAS-*hiw*ΔRING (Wu et al., 2005) contains in addition to the mutations carried by UAS-*hiw*, mutations in the first two cysteine residues (C4991 and C4994) in the RING finger domain required for *hiw* ubiquitin ligase function. Other fly stocks used were three independent UAS-hiw^{RNAi} lines –v26998 (III), v28163 (II), and v36085 (II) from Vienna *Drosophila* RNAi Center. Flies were raised on standard maize meal food at 18°C.

Behavioral assays

Flies were tested in both the sedation and recovery assays as described previously (Wen et al., 2005) but with slight modifications. In brief, 20 active and well fed males were selected under CO_2 anesthesia and allowed to recover for 24 h before use. For the sedation assay, 1 mL ethanol solution at 50% concentration was added to a piece of folded Kimwipe tissue (11.4 cm × 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 and scored in percentage. 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 min multiplied by the time of sedation in minute and divided by the total number of flies sedated as given by this equation: $\mathrm{MST} = \sum x_t \times t/N$ where x_t is the number of flies sedated at a given sedation time t and N the total number of flies sedated.

For the recovery assay, flies were exposed to ethanol vapor for 12 min in a vial closed with a cotton wool plug, to which 1 mL of 100% ethanol was added slowly to allow ethanol to soak into the plug. After this exposure, all flies tested remained motionless at the bottom of the vial. Subsequently, the ethanol-soaked cotton plug was then replaced with a fresh ethanol-free cotton plug. The number of flies recovered from ethanol sedation as shown by their climbing and flying activities were counted at 3-min intervals and scored in percentage. The mean recovery time (MRT) used as a measure of fly's ability to recover from the sedative effects of ethanol was calculated as the sum of the number of flies recovered at every 3 min multiplied by the time of recovery in minute and divided by the total number of flies recovered as given by this equation: $\mathrm{MST} = \sum x_t \times t/N$ where x_t is the number of flies recovered at a given recovery time t and N the total number of flies recovered.

Ethanol absorption and metabolism

Flies internal ethanol compositions were determined from whole fly homogenates of 2 flies per samples using an Alcometer. To study ethanol absorption, flies were exposed to 100% ethanol vapor for 12 min in the recovery assay 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 homogenized in 20 μL PBS buffer, pH 7.4. The homogenate was then centrifuged at 12000 r/min at 4°C for 10 min, and the supernatant was collected. The ethanol concentration in the supernatant was measured using the Alcometer.

Confocal microscopy

Confocal microscopy was performed using a laser scanning confocal system for verifying GFP expression in the adult *Drosophila* brain neurons. 2–5 days old adult *Drosophila* brains were imaged on the confocal microscope and images were scanned using excitation (480 nm) and detection (500–550 nm) filters. The gain was chosen as the maximum gain that did not saturate the signal for each sample studied. A complete z-stack was acquired for each brain sample. Tissues were studied at X20.

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

Ethanol sensitivity in *Drosophila*

When flies are exposed to ethanol vapor, they display several behavioral changes: they become hyperactive, uncoordinated and eventually sedated. Following the cessation of ethanol administration, flies require some length of time to recover from the sedative effects of ethanol. These two behavioral phenomena i.e. one that leads to ethanol sedation and the period of recovery from the sedative effects of ethanol can be

quantified using the well established sedation and recovery assays (Wen et al., 2005). These two assays were modified and used in this study. First, to determine the most appropriate dose of ethanol to use in the sedation assay, an ethanol-dose response test was performed using the wild-type stock (WT). Ethanol solutions of 10%, 30%, 40%, 50% and 80% were made by mixing ethanol and water at the ratio (vol/vol) of 1:9, 3:7, 4:6 and 8:2 respectively and used to measure the fly's resistance to the sedative effect of ethanol (see Methods). Flies displayed behavioral changes when exposed to the appropriate dose of ethanol as noted earlier. Naive wild-type flies exposed to 100% ethanol vapor showed a mean sedation time (MST) of 20.5±0.2 min, whereas it took longer with 80% ethanol (MST 32.3±0.5 min), 50% ethanol (MST 53.3±0.6 min) and 40% ethanol (MST 61.7±0.4 min) (Fig. 1A, 1B). The data for 10% and 30% ethanol vapor concentrations were not included as it took a very long time for the flies exposed to these concentrations to become sedated. As expected, sedation was dose dependent, such that lower ethanol concentration resulted in longer MSTs (Fig. 1B). A 100% or 80% dose gives a sharp, clear peak of activity but has a short lived reaction, suggesting that any alterations in sensitivity might be difficult to quantify because of fast immobilisation and the fact that these concentrations may be deleterious to the flies. At 50% ethanol concentration, it takes a reasonable amount of time to sedate the flies and it is expected (based on the profiles) that small differences in sedation time could be detected at this concentration. A 40% ethanol concentration also offers alterations in sensitivity that might easily be measured but the MST is rather long. Thus, 50% ethanol concentration was chosen and used in the subsequent sedation protocol described in this article.

For the recovery assay and using 100% ethanol concentration (see Methods for a detailed description of the assay), naive wild-type flies reproducibly recovered fully from ethanol sedation after 36 min corresponding to an average MRT of 24±0.7 min (Fig. 1C and data not shown).

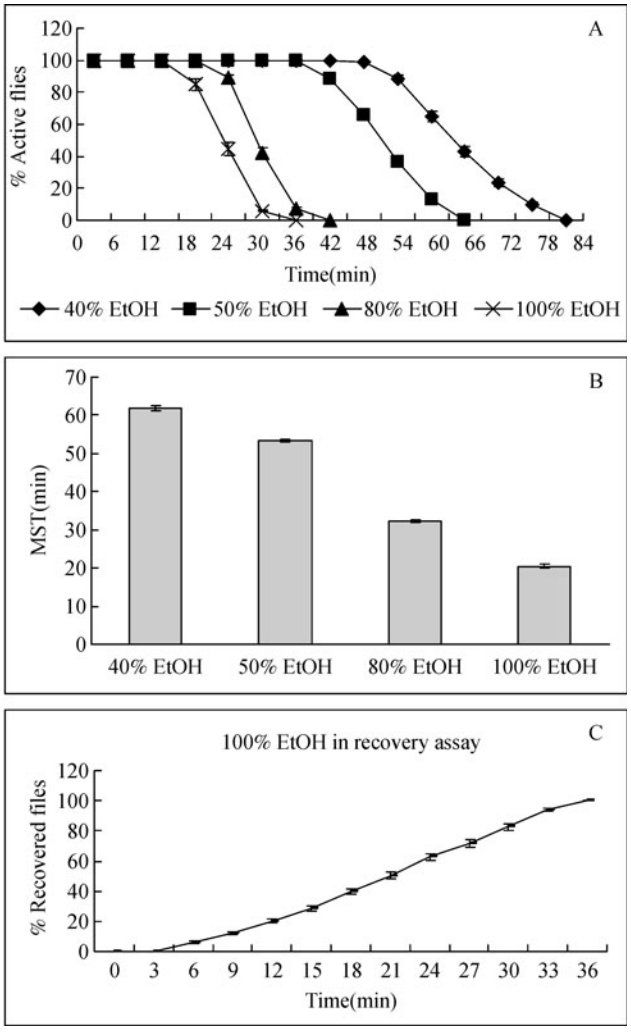

Figure 1 Effects of ethanol dose on male wild-type (WT) control (Ctl) flies. (A) The duration of activities of flies undergoing sedation correlates with ethanol dose of 40%, 50%, 80% and 100% as indicated. For these and all subsequent sedation curve data, the percentage of flies scored as active during sedation in a group of 20 male flies is graphed as a function of time. (B) Mean sedation time (MST) for experiment represented in (A). A one-way ANOVA analysis revealed significant main effects of ethanol dose across the various concentrations used ($P < 0.0001$). (C) The duration of activities of WT flies in the recovery assay. One-way ANOVA revealed no significant difference within repeats, $n = 18$. Error bars represent the s.e.m; $n = 20$ in all ethanol (EtOH) concentrations and corresponds to the number of experiments, not the number of flies.

Normal ethanol sensitivity requires *hiw* function in *Drosophila*

To investigate the possible involvement of Hiw in the behavioral sensitivity to ethanol, this study assessed the sedative effect of ethanol on Hiw-deficient flies (Wan et al., 2000; Wu et al., 2005). Three independently isolated *hiw* alleles were used (Fig. 2A). The mutant strain, EP1305, showed increase in sensitivity in response to ethanol sedation and recover significantly slower when compared to the control flies (Fig. 2B), suggesting that a functional *hiw* gene is required for the normal sensitivity to ethanol. This strain is located at the 3′ end of the large intron which contains two genes, *CG5541* and *CG3594* (Fig. 2A; Wu et al., 2005). It is thus possible that hiw^{EP1305} also disrupt these two genes in the *hiw* intron, thereby causing the observed sensitivity. In addition, it is also possible the hiw^{EP1305} carries a second unidentified mutation or allelic variant(s) that leads to the ethanol sensitivity phenotype. To clarify these, two other independently isolated alleles of *hiw* (*ND8* and *EP1308*) were tested in the 2 behavioral paradigms for ethanol sensitivity. The hiw^{ND8} is a nonsense allele expressing truncated protein (Wu et al., 2005) and has been shown to behave like a functional loss-of-function allele (Wu et al., 2005) while the hiw^{EP1308} allele is a *P* element insertion mapping to the 5′ untranslated region of the first exon (Wan et al., 2000). These

two alleles are much less likely to disrupt the two genes in the *hiw* intron (see Fig. 2A). Interestingly, both of these alleles showed increased sensitivity in response to the sedative effect of ethanol and their rate of recovery from ethanol sedation were significantly slower than that of the control flies (Fig. 2B). Importantly, a similar difference to wild-type was observed when female *hiw* flies for all these mutants were tested in the two behavioral assays (Fig. 2B). These data also show that global perturbation of Hiw signaling in adult *Drosophila* increase ethanol sensitivity. Further, analysis of the ethanol absorption and concentration curve of null allele hiw^{ND8} and wild-type flies revealed no significant differences between the two strains (Fig. 2C). The enhanced sensitivity to ethanol is, therefore, likely due to changes in the sensitivity of the central nervous system rather than to changes in pharmacokinetic or metabolic factors.

To prove that Hiw regulates ethanol sensitivity by acting directly in the nervous system, 3 independently isolated UAS-hiw^{RNAi} lines were used to silence *hiw* expression in the presence of elav-GAL4 line which drives expression in the whole nervous system (Fig. 3A, 3C). The elav-GAL4 line displayed increased ethanol sensitivity in the presence of P[UAS-hiw^{RNAi}] transgene (Fig. 3C). Neither the elav-GAL4 driver nor the P[UAS-hiw^{RNAi}] transgene alone altered the ethanol sensitivity seen Fig. 3C. Thus, the destruction of *hiw* transcriptional unit increased susceptibility to ethanol sedation. Taken together, this confirms that *hiw* function is required for normal ethanol sensitivity in *Drosophila*.

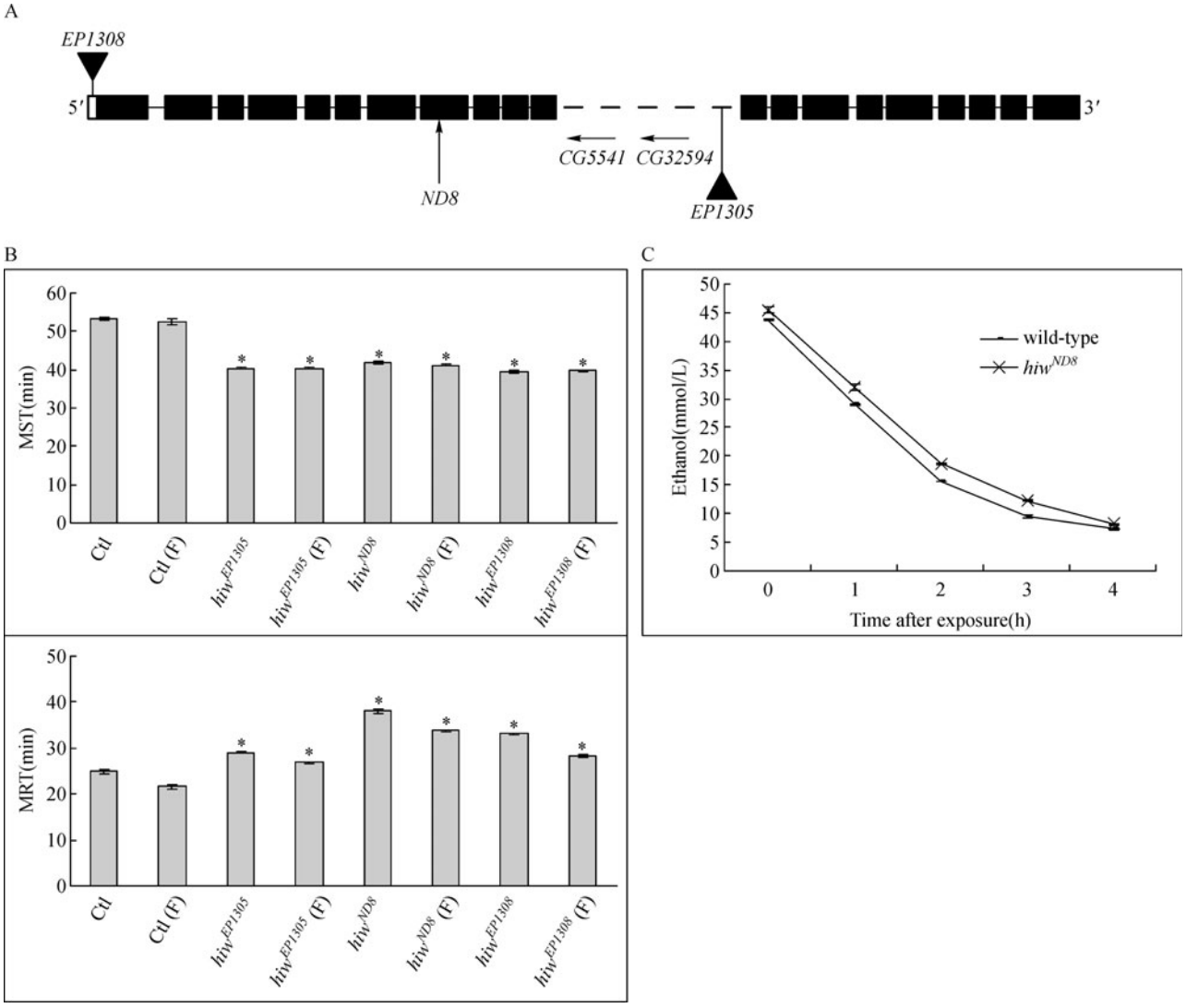

Figure 2 Hiw-deficient flies have abnormal ethanol sensitivity phenotype. (A) Genomic structure of *hiw* alleles. Exons are designated in rectangles, with translated regions in closed boxes (shaded black) and un-translated regions in open box (white box). Two genes, *CG5541* and *G32594*, are located in large intron in dashed line (http://www.flybase.com; Wan et al., 2000; Wu et al., 2005). (B) Upper and lower panels show ethanol sensitivity measured as mean sedation time (MST) and mean recovery time (MRT) respectively. Asterisk, $P < 0.01$; n = 6–20. (C) Ethanol concentrations after 12 min exposure to ethanol vapor are shown. No significant difference was seen between hiw^{ND8} and wild-type control (Student's t-test; n = 3). In all figures, n = number of experiments, not the number of flies, and error bars indicate s.e.m.

This study also wished to determine whether an increase in the endogenous levels of Hiw expression in all neurons could lead to resistance. This required that flies overexpressing Hiw proteins in the nervous system be assayed for ethanol sensitivity. Accordingly, flies carrying one wild-type copy each of UAS-*hiw* transgene and elav-GAL4, and UAS-*hiw*ΔRING transgene and elav-GAL4 were tested for ethanol sensitivity in the sedation and recovery assays (Fig. 3D). The *hiw*ΔRING flies are identical to wild-type *hiw* with the exception of two additional mutations in the first two cysteine residues (C4991 and C4994) in the RING finger domain. These residues have been shown to be required for *hiw* ubiquitin ligase function (Wu et al., 2005). Overexpression of Hiw in the nervous systems led to resistance to the sedating effects of ethanol and a shorter recovery time. Overexpression of *hiw*ΔRING has no significant effect on ethanol sensitivity (Fig. 3D), indicating that the ubiquitin ligase function of Hiw mediates the ethanol response. This raises the possibility that *hiw* functions in cooperation with an ubiquitin ligase in the presence of ethanol and may be involved in the ubiquitination of ethanol induced denatured proteins via an ubiquitin-proteasome system (UPS).

A recent study has identified the neuronal networks in the ellipsoid body as essential mediator of the behavioral response to ethanol (Urizar et al., 2007). To test whether the ethanol sensitivity phenotype seen with *hiw* mutants might be

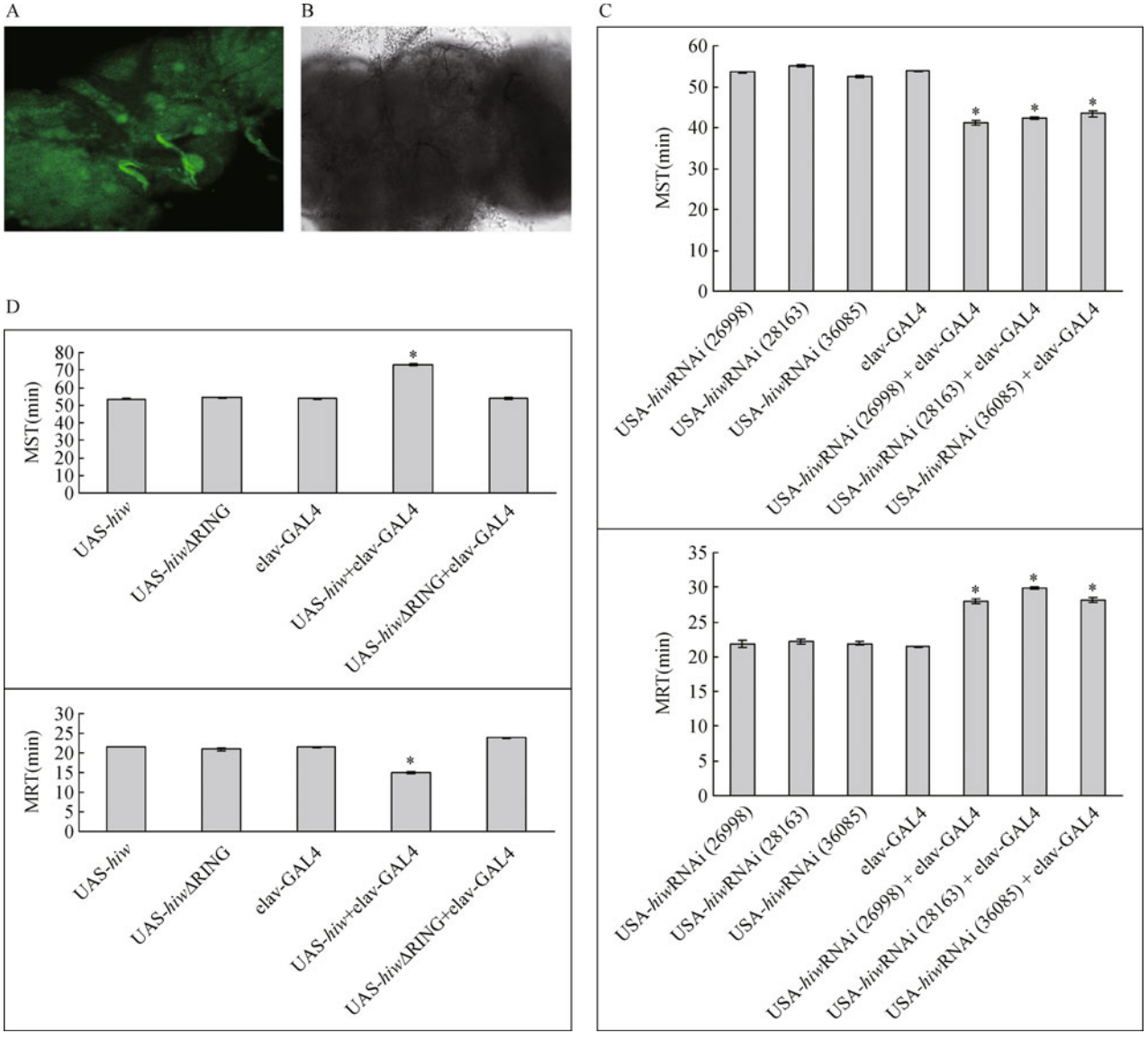

Figure 3 Transgenic expression of Hiw in the whole nervous system alters ethanol sensitivity in the sedation and recovery assays. (A) A confocal microscope image of adult brain of a fly carrying elav-GAL4 and UAS-GFP-Hiw. Expression of GFP is green. (B) A negative control showing the adult brain of a fly carrying only the elav-GAL4 vector. (C and D) Upper and lower panels show ethanol sensitivity measured as mean sedation times (MSTs) and mean recovery times (MRTs) respectively. Asterisk, $P < 0.01$; the three RNAi stock lines are indicated in bracket. In all figures, $n = 5$, and error bars indicate s.e.m.

mediated by the ellipsoid body neurons, the 3 independently isolated UAS-*hiw*^{RNAi} lines were used to silence *hiw* expression in the presence of c819-GAL4 line which drives expression in the R2/R4m ring neurons of ellipsoid body (Fig. 4A, 4B). Consistent with a role for *hiw* in the nervous system, the P[c819-GAL4] line displayed increased ethanol sensitivity in the presence of UAS-*hiw*^{RNAi} transgene (Fig. 4B lower panel). Neither the c819-GAL4 driver nor the P[UAS-*hiw*^{RNAi}] transgene alone altered the ethanol sensitivity (Fig. 4B lower panel). It should however, be noted that driving the RNAi construct with c819-GAL4 caused enhanced ethanol sensitivity measured in the recovery assay (Fig. 4B lower panel) but not in the sedation assay (Fig. 4B upper panel). This raises the possibility that this region of the brain regulates the ability to recover from ethanol sedation but not susceptibility to ethanol sedation. Given this results, it was tested whether excess Hiw in an otherwise wild-type background could lead to a reduced sensitivity to ethanol in the neural structure. Overexpression of either of transgenic *hiw* or transgenic *hiw*ΔRING from c819-GAL4 did not lead to any overt ethanol sensitivity phenotype in both assays (Fig. 4C), suggesting that sensitivity is not affected by a raised Hiw level in this structure.

Discussion

To obtain a better understanding of the molecular mechanisms underlying the effect of ethanol in the nervous system, this study modified and characterized sedation and recovery assays (Wen et al., 2005)–the two independent assays for measuring sensitivity to the sedative effects of ethanol in *Drosophila*. Using these assays, this study showed a component of a ubiquitin system, the E3 ubiquitin ligase Hiw mediating fly sensitivity to acute dose of ethanol. Because RNAi functional knockdown of *hiw* expression in the nervous system mimic this phenotype, such behavior is attributed to the destruction of *hiw* expression in the *hiw* mutants flies. Interestingly, increased levels of Hiw in the whole nervous system lead to reduced sensitivity to ethanol. Thus, this study supports the hypothesis that *Drosophila* Hiw regulates ethanol sensitivity by acting within the nervous system. In addition, this study has shown that *hiw* silencing in the ellipsoid body neurons regulates distinct aspects of the behavioral response to ethanol and in this case recovery from ethanol sedation.

Previous studies have mapped ethanol sensitivity to specific brain regions in *Drosophila* including the R2/R4m neurons in the ellipsoid body (Urizar et al., 2007) and subset of neurons in the central complex (Rodan et al., 2002). The R2/R4m neurons in the ellipsoid body have been implicated in NMDA-receptor dependent olfactory long-term memory consolidation (Wu et al., 2007) and in visual pattern memory (Pan et al., 2009). The present study has shown that sensitivity to ethanol is a neuronal phenotype that is mediated by the Hiw ubiquitin-ligase domain in flies. This study also implicates the ellipsoid body region of the brain in ethanol sensitivity and suggests that *hiw* plays a role at least in this tissue. Though, the exact role of the ellipsoid body neurons in Hiw-mediated ethanol sensitivity is not known, we can infer from previous studies on the larval neuromuscular junction (NMJ) (Wan et al., 2000, Wu et al., 2005) that Hiw (through its functional RING finger domain) may mediate synaptic signaling in these neurons. Thus, Hiw-dependent regulation of synaptic morphology may be lost in flies with reduced levels of *hiw* in these neurons and this loss leads to an increase in ethanol sensitivity measured in the recovery assay. In addition, the fact that olfactory learning and ethanol sensitivity appears to share similar molecular mechanism (Cheng et al., 2001) may indicate a role for Hiw in some forms of memory consolidation. With the RNAi result that indicated a necessary role of ellipsoid body neurons, it could only be suggested at this time that the ellipsoid body neurons may be a brain region where Hiw functions to affect ethanol sensitivity measured in the recovery assay. Nevertheless, further experiment is required to confirm the role of this brain region in Hiw-mediated ethanol sensitivity.

Exposure of both mammals and flies to varying concentrations of ethanol has been reported to have distinct behavioral consequences (Rodan et al., 2002). In *Drosophila*, these can be separated using different assays such as inebriometer and locomotor tracking system (Rodan et al., 2002), inebriometer and recovery assays (Berger et al., 2004). Using sedation and recovery assays this study has also shown that RNAi-mediated functional knockdown of *hiw* under the control of c819-GAL4 driver, which did not affect sensitivity to the sedative effects of ethanol in the sedation assay, altered sensitivity to the recovery from ethanol sedative effect. Thus, this present study has further established that genetic dissociation actually exists between assays quantifying different aspects of ethanol responses.

Of particular interest is the role of the E3 ubiquitin ligase domain of Hiw in kinase signaling pathway. Studies have shown that Hiw E3 ubiquitin ligase restricts synaptic growth primarily by downregulation of Wallenda (Wnd), a MAP kinase kinase (Collins et al., 2006). It is likely that this domain plays an important role in the neuroadaptation underlying ethanol-induced behavior by downregulation of Wnd because *highwire; wallenda* double mutant (*hiw*^{ND8}; *wnd*^{1}/*wnd*^{2}) have been shown to completely suppress the *highwire* synaptic overgrowth phenotype (Collins et al., 2006). *Wnd* has been shown to be essential for synaptic overgrowth caused by both overexpression of a ubiquitin hydrolase and a loss of a ubiquitin ligase and, the gene is reported to behave like a candidate substrate for ubiquitination that could mediate synaptic overgrowth (Collins et al., 2006), suggesting a model that *wallenda* is denatured and degraded in the presence of ethanol and that the ubiquitin ligase function in *hiw* is involved in the specific degradation of abnormal *wallenda* proteins. A prediction of this model is

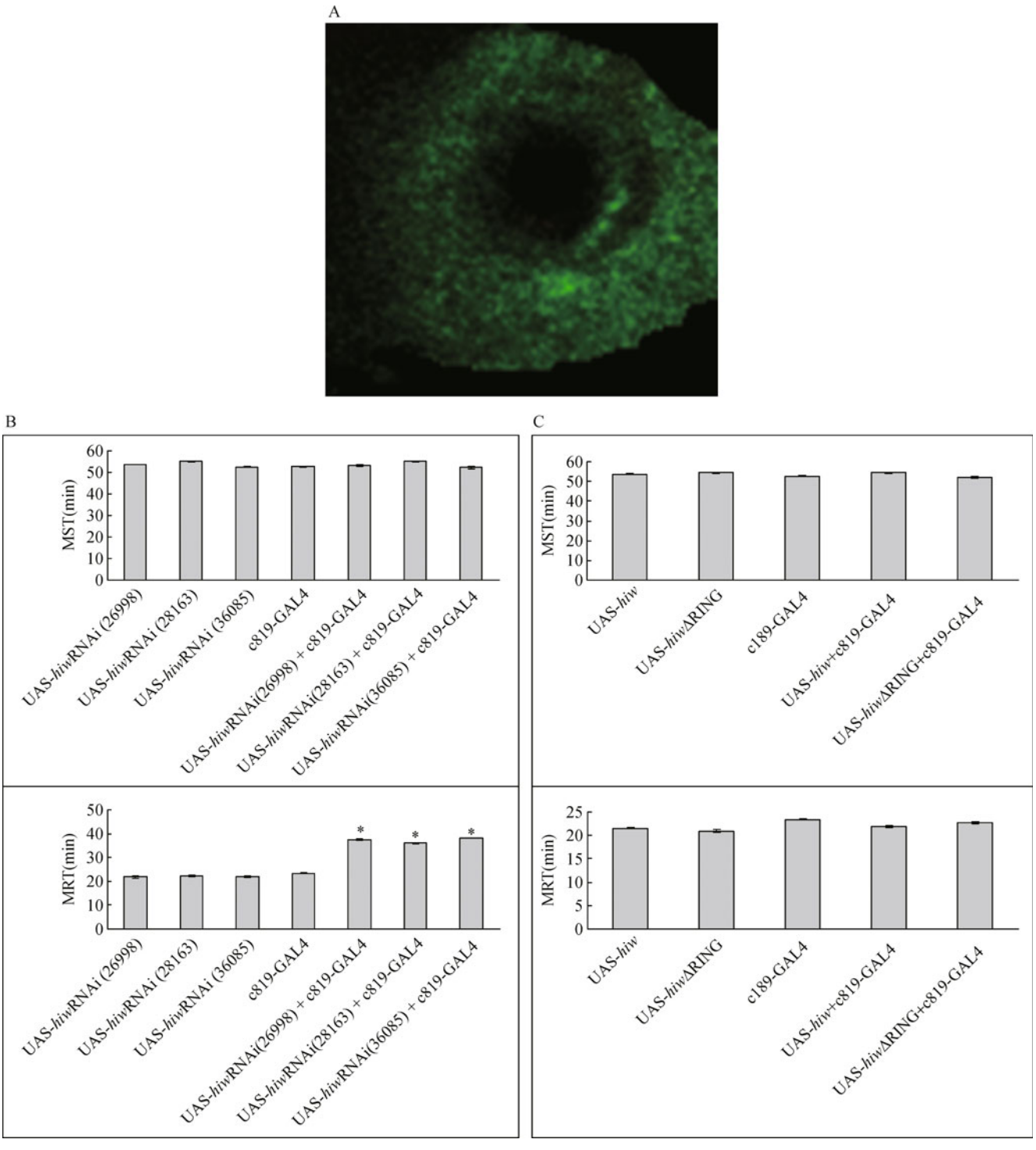

Figure 4 RNAi expression of Hiw in the ellipsoid body neurons of the brain alters ethanol sensitivity only in the recovery assay, while overexpression of Hiw in this region showed no significant alterations in ethanol sensitivity in both the sedation and recovery assays. (A) A confocal microscope image of adult brain of a fly showing P[GAL4] directed expression of GFP in the R2/R4m ring neurons of ellipsoid body (c819). Expression of GFP is green. (B and C) Upper and lower panels show ethanol sensitivity measured as mean sedation times (MSTs) and mean recovery times (MRTs) respectively. Asterisk, $P < 0.01$; the three RNAi stock lines are indicated in bracket. In all figures, $n = 5$ and error bars indicate s.e.m.

that *highwire; wallenda* double mutant (hiw^{ND8};wnd^{1}/wnd^{2}) will exhibit normal ethanol sensitivity.

In summary, this study showed that normal function of *hiw* is necessary for the *D. melanogaster* to show normal sensitivity to the sedative effects of ethanol. The result suggests that the human homolog of *hiw* gene, *Pam* may also

effectively affect sensitivity to ethanol sedation, providing a new direction for research into alcohol dependency.

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