Cell

Distinct Behavioral Responses to Ethanol Are Regulated by Alternate RhoGAP18B Isoforms

Adrian Rothenfluh,[1,*] Robert J. Threlkeld,[1] Roland J. Bainton,[2] Linus T.-Y. Tsai,[1,3] Amy W. Lasek,[4] and Ulrike Heberlein[1,3,4,*]
[1] Department of Anatomy
[2] Department of Anesthesia
[3] Program in Neuroscience
University of California, San Francisco, San Francisco, CA 94143, USA
[4] Ernest Gallo Clinic and Research Center, Emeryville, CA 94608, USA
*Contact: adrian.rothenfluh@ucsf.edu (A.R.), ulrike.heberlein@ucsf.edu (U.H.)
DOI 10.1016/j.cell.2006.09.010

SUMMARY

In most organisms, low ethanol doses induce increased activity, while high doses are sedating. To investigate the underlying mechanisms, we isolated *Drosophila* mutants with altered ethanol responsiveness. Mutations in *white rabbit* (*whir*), disrupting *RhoGAP18B*, are strongly resistant to the sedating effects of ethanol. This resistance can be suppressed by reducing the levels of Rho1 or Rac, implicating these GTPases in the behavioral response to ethanol. Indeed, expression of constitutively active forms of Rho1 or Rac1 in adult flies results in ethanol resistance similar to that observed in *whir* mutants. The *whir* locus produces several transcripts, *RA–RD*, which are predicted to encode three distinct RhoGAPs that share only the GAP domain. The *RC* transcript mediates the sedating effects of ethanol, while the *RA* transcript regulates its stimulant effects. Thus, distinct RhoGAPs, encoded by the same gene, regulate different manifestations of acute ethanol intoxication.

INTRODUCTION

Alcohol addiction is a devastating and widespread social and medical problem influenced by both genetic and environmental factors (Devor and Cloninger, 1989; Enoch and Goldman, 1999; Schuckit, 2000). Despite extensive efforts, the conclusive molecular identification of its genetic risk factors has met with limited success. Part of the difficulty arises from the fact that the genetic risk factors are complex and heterogeneous and that overall assessment of the "alcoholic phenotype" is based on a broad set of characteristics that are likely under different genetic regulation (Cloninger, 1987). For this reason, some studies have focused on more discrete and measurable phenotypes that are commonly associated with alcohol addiction, such as the "level of response" to a specific ethanol dose delivered in a laboratory. Multiple studies have found that a reduced response to the acute intoxicating effects of ethanol is correlated with an increased risk for alcoholism (Schuckit et al., 2004). Similar observations have been made in rodent models: Genetic manipulations that cause a reduced response to the sedating effects of ethanol also commonly lead to increased ethanol self-administration (Thiele et al., 1998), and vice versa (Hodge et al., 1999). Therefore, studying the genetic factors contributing to a relatively simple response to ethanol should in turn provide valuable clues about the more complex process of addiction.

Drosophila melanogaster has been developed as a useful model system to define molecules and signaling pathways mediating the acute intoxicating effects of ethanol (Guarnieri and Heberlein, 2003). Behaviors induced by acute exposure in *Drosophila* are very similar to those observed in mammals: Low ethanol doses induce a state of increased activity, while higher doses are sedating (Singh and Heberlein, 2000; Parr et al., 2001; Wolf et al., 2002). Moreover, homologous genes have been implicated in these responses in both flies and mice, including the regulatory subunit of protein kinase A (Park et al., 2000; Thiele et al., 2000), calcium/calmodulin-sensitive adenylate cyclases (Moore et al., 1998; Maas et al., 2005), and neuropeptide Y (Thiele et al., 2004; Wen et al., 2005). Therefore, unbiased genetic screens for *Drosophila* mutants with altered responses to the acute intoxicating effects of ethanol will likely identify valuable candidate genes to be studied in mammalian models and humans. Here we describe the characterization of mutations in the *Drosophila RhoGAP18B* gene, isolated due to their strong resistance to the sedating effects of ethanol.

Small GTPases of the Rho family act as molecular switches transducing extracellular signals to changes in

the actin cytoskeleton (Etienne-Manneville and Hall, 2002; Meyer and Feldman, 2002), playing important roles in regulating nervous-system development and its mature plasticity (Bonhoeffer and Yuste, 2002; Luo, 2002; Sin et al., 2002; Carlisle and Kennedy, 2005). The activity of Rho GTPases is regulated positively by guanine nucleotide exchange factors (GEFs) and negatively by GTPase-activating proteins (GAPs). In the *Drosophila* nervous system, these proteins have been implicated in neuroblast proliferation; axon guidance, growth, and branching; and dendrite morphogenesis (Lee et al., 2000, 2003; Hakeda-Suzuki et al., 2002; Scott et al., 2003; Ng et al., 2002). While mutations in the GTPases cause severe pleiotropic defects (Lee et al., 2000), mutations affecting GAPs or GEFs result in more restricted phenotypes (Billuart et al., 2001; Lundstrom et al., 2004). This is probably due to the fact that the activity of Rho GTPases is regulated by a large number of GEFs and GAPs (Johndrow et al., 2004). The specificity of Rho GTPase activity is thus likely imparted by the specific regulatory GEFs and/or GAPs.

The *RhoGAP18B* locus encodes four transcripts, *RA–RD*, which in turn are predicted to encode three proteins that share only the conserved GAP domain. We show that distinct RhoGAP18B isoforms mediate different aspects of the flies' response to ethanol: hyperactivity and sedation. These distinct behavioral effects are mediated by *RhoGAP18B* function in the same subset of adult CNS neurons. Thus, different protein isoforms encoded by a single gene can function in the same group of cells to regulate distinct behavioral outputs.

RESULTS

white rabbit Mutants Show Resistance to Ethanol-Induced Sedation

When exposed to a relatively high concentration of ethanol vapor, flies initially display a period of increased locomotor activity, which is followed by sedation (Figure 1A). These changes in behavior can be monitored with a locomotor tracking system, which determines the velocity of movement (Wolf et al., 2002), and a loss-of-righting (LOR) test, which quantifies the degree of sedation. To identify genes involved in the behavioral response to ethanol, we screened a collection of strains carrying P element insertions for alterations in ethanol-induced locomotor behavior. We isolated multiple mutants carrying insertions in an X-linked gene that we named *white rabbit* (*whir*) for its diverse role in regulating responses to abused drugs as described in the song "White Rabbit" by Jefferson Airplane. *whir* mutants showed resistance to ethanol-induced sedation measured with either the locomotor tracking system (Figure 1A) or the LOR test (Figure 1B). All *whir* alleles tested were recessive and failed to complement each other in the LOR assay (Figure 1C).

Ethanol absorption was normal in *whir* flies (see the Supplemental Data available with this article online). In addition, the mutant flies performed normally in various other behavioral assays, including those measuring sensitivity to CO_2-induced sedation, nicotine toxicity, spontaneous locomotion, circadian rhythms, and negative geotaxis (Figure S1). However, *whir* mutant flies also showed increased resistance to the acute effects of nicotine and cocaine (Figure S2). In addition, the strongest allele, $whir^3$, showed reduced viability (of the stock; individual flies surviving through development appeared normal), while the $whir^1$ and $whir^2$ alleles were fully viable.

white rabbit Mutants Disrupt *RhoGAP18B*

The behavioral phenotype of *whir* mutants segregated with the P element insertion after five generations of outcrossing to wild-type strains. In addition, multiple precise excision strains generated from both $whir^1$ and $whir^3$ showed wild-type behavior (Figure 1B), confirming that the P element insertions were responsible for the behavioral defects. Inverse PCR and DNA sequence analysis of the $whir^1$ and $whir^2$ alleles revealed that the P elements were inserted in the open reading frame (ORF) of *CG7502* (hatched exons in Figure 2A), a gene predicted by the Berkeley Drosophila Genome Project to be located within an intron of the *RhoGAP18B* gene. However, *CG7502* is in fact part of *RhoGAP18B* since sequencing of two cDNAs (corresponding to ESTs *RE42510* and *SD23384*) containing the 5′ end of *CG7502* revealed that they also contained downstream exons of *RhoGAP18B*. The existence of this transcript, *RD*, was confirmed by northern blots and RT-PCR using mRNA isolated from adult fly heads and bodies (data not shown). We also used RT-PCR analysis and cDNA sequencing to confirm the existence of the *RhoGAP18B RA* transcript (Figures 2A and 2C). Finally, we were unable to detect transcripts encompassing the first exon of *RA* and any of the predicted *CG7502* exons, indicating that *RhoGAP18B* is transcribed from at least two promoters separated by approximately 11 kb.

Extensive additional transcript analysis revealed the existence of four *RhoGAP18B* transcripts: the originally predicted (and now confirmed) *RA* transcript; a splice variant *RB*, which lacks 519 bases in the 5′UTR of *RA*; and two additional transcripts, *RC* and *RD*, which contain sequences originally ascribed to *CG7502*, in addition to *RhoGAP18B*. The three predicted proteins, RhoGAP18B-PA, -PC, and -PD, share the Rho-family GAP domain, which is encoded in the last two exons, but they differ extensively in their N termini (Figure S3). The function of these different predicted RhoGAP18B proteins is likely important, as their presence and sequence is highly conserved in *Drosophila pseudoobscura* (Figure S3), a species that diverged from *Drosophila melanogaster* approximately 30 million years ago.

Loss of *RC* Transcript Correlates with Ethanol Resistance

The P elements in $whir^1$ and $whir^2$ are inserted 5′ of the *RD* transcription start site, in the ORF of the *RC* transcript. In $whir^1$ mutant flies, the 5.5 kb *RC* transcript was undetectable, while the 3.9 kb *RD* transcript was still present (Figure 2B). Because the abundance of the 2.1 kb *RA*

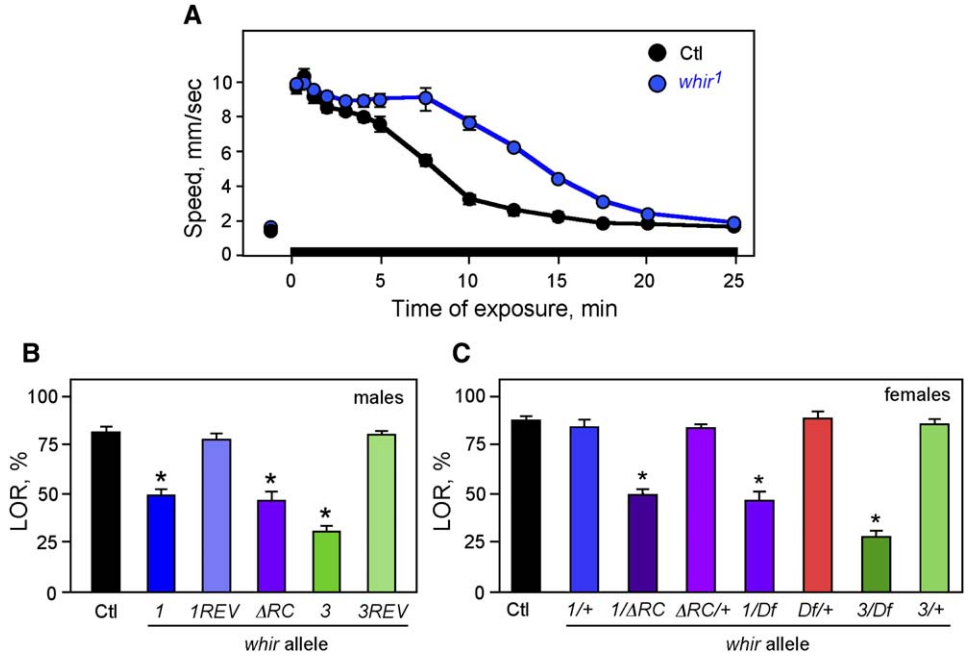

Figure 1. *white rabbit* Flies Show Resistance to Ethanol-Induced Sedation

In this and all other figures, error bars indicate means ± SEM.

(A) Locomotion video tracking of groups of flies exposed to a high concentration of ethanol vapor (110/40 ethanol vapor/humidified air [E/A] flow rate); exposure starts at time 0. Flies show an initial increase in locomotion that is followed by gradual sedation, reflected in a reduction in the speed of locomotion. Compared to control flies (Ctl), *whir*^{1} mutant flies show a delay in sedation (n = 4 experiments).

(B and C) Loss of righting (LOR) after 26 min of ethanol exposure (110/40 E/A). LOR refers to the inability of flies to regain upright posture upon mechanical stimulation. Note that although all flies stop moving after 26 min of exposure, not all have lost their righting reflex. *whir*^{1}, *whir*^{3}, and *whir*$^{\Delta RC}$ flies show significantly reduced LOR compared to control flies. Precise excision (*REV*) of the P element from both *whir*^{1} and *whir*^{3} flies restores normal ethanol sedation (B). All mutant *whir* alleles are recessive and fail to complement each other or a deficiency uncovering the *whir* gene (C) (*$p < 0.001$, Tukey's HSD test, n = 5–9 experiments). The *whir*^{3} phenotype is significantly stronger than *whir*^{1} or *whir*$^{\Delta RC}$ ($p < 0.005$); similarly, the phenotype of *whir*^{3}/*Df* is stronger than that of *whir*^{1}/*Df* ($p < 0.002$).

transcript was very low in adult head extracts (Figure 2B), we designed specific primer pairs for quantitative RT-PCR analysis of the *RA*, *RC*, and *RC*+*RD* transcripts (*RD* overlaps completely with *RC*; these transcripts cannot be distinguished by RT-PCR) in adult head RNA. A large reduction of RA and RC was observed in *whir*^{1} flies (Figure 2C), suggesting that a deficit in either or both of these transcripts may cause the observed ethanol-resistance phenotype. To distinguish these possibilities, we generated a mutant, *whir*$^{\Delta RC}$, that specifically disrupts the *RC* transcript by imprecise excision of the *whir*^{1} P element. This mutant contains a 625 bp deletion in the first *RC* exon (Figure 2A), which is predicted to cause an early termination of the *RC* ORF. *whir*$^{\Delta RC}$ flies contain normal levels of *RA*, *RC*, and *RC*+*RD* transcripts (Figure 2C), and the only difference from wild-type is a shortened *RC* transcript (as expected from the genomic DNA deletion; Figure 2D). Thus, *whir*$^{\Delta RC}$ flies should produce the RhoGAP18B-PA and -PD proteins but lack PC. *whir*$^{\Delta RC}$ mutants showed resistance to ethanol-induced sedation identical to that observed with *whir*^{1} and also failed to complement *whir*^{1}'s phenotype (Figures 1B and 1C), strongly suggesting that the loss of the *RC* transcript and PC protein results in resistance to the sedating effects of ethanol.

RhoGAP18B Stimulates GTPase Activity of Rho-Family GTPases In Vitro

To determine whether *RhoGAP18B* encodes an active GAP protein, we expressed a 245 amino acid fragment containing the GAP domain as a GST-fusion protein in bacterial cells (see Experimental Procedures). This fusion protein enhanced the intrinsic GTPase activity of human Rac and Cdc42, but not RhoA (or the negative control Ras; Figure 3A). The same result was obtained for full-length RhoGAP18B-PA, encoded by the *RA* transcript (data not shown); full-length PC protein was insoluble and therefore could not be tested. These data show that *RhoGAP18B* encodes proteins with GAP activity.

Involvement of Rho-type GTPases in Ethanol-Induced Sedation

The loss of RhoGAP18B-PC function in *whir* mutant flies is expected to cause excessive activity of one or more small GTPases of the Rho superfamily. If this is indeed the case, reducing the levels of these GTPases should suppress the

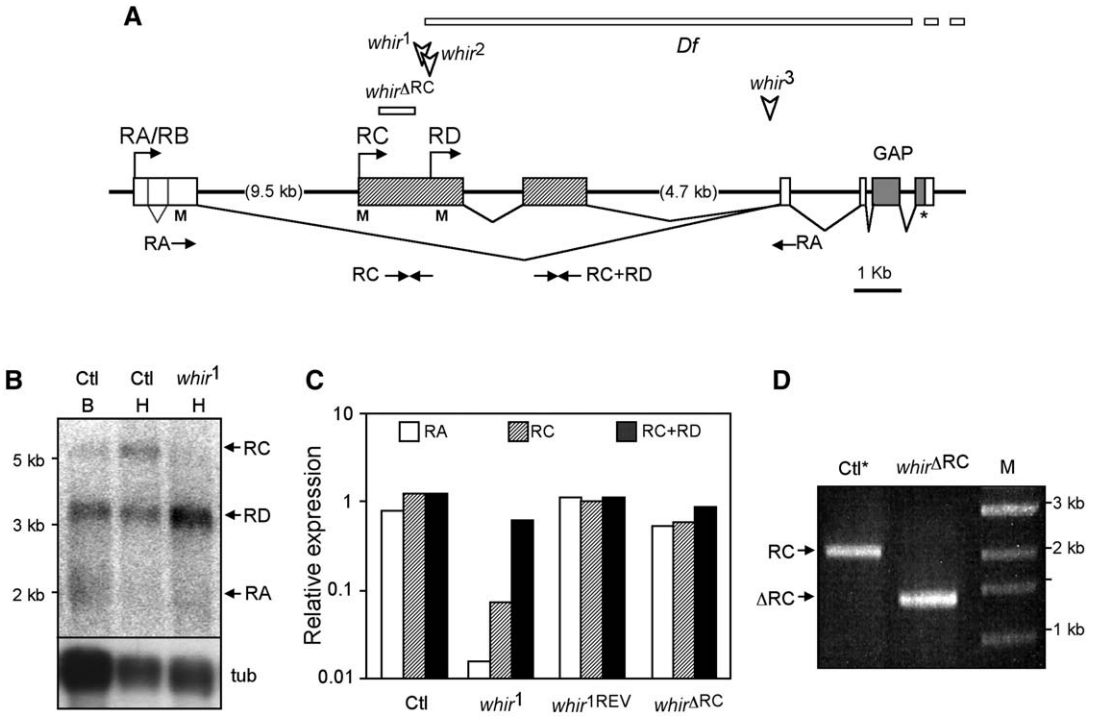

Figure 2. *white rabbit* Encodes RhoGAP18B

(A) Schematic representation of the *RhoGAP18B* locus, encompassing ~30 kb of genomic DNA. Exons are shown as boxes and introns as lines. The transcription start sites for the *RA*/*RB*, *RC*, and *RD* transcripts and the position of predicted translation initiation codons (M) and the termination codon (*) are shown. The shared sequence encoding the GTPase-activating domain (GAP) is highlighted in gray, while the hatched exons represent *CG7502*, a gene previously predicted to lie within the large *RhoGAP18B* intron. P element insertion sites are represented by triangles, the ΔRC deletion is indicated by a box, and sequences deleted by the deficiency (*Df*) are indicated by a bar. PCR primers used in (C) are indicated with arrows below the gene structure. *RA* and *RB* share their transcription start site and do not differ in their predicted open reading frame; *RB* is produced by splicing of a 519-base intron located in the 5′UTR of *RA* (gray lines).

(B) Northern blot probed with a fragment just 5′ of the GAP domain that is common to all transcripts. mRNA was isolated from heads (H) and bodies (B) of control (Ctl, corresponds to the w^{1118} Berlin genetic background) and $whir^1$ flies. The *RC* transcript is more abundant in wild-type heads than bodies and is undetectable in heads of mutant $whir^1$ flies. The ~2 kb *RA* signal is weak on northern blots, and the band is also less sharp, possibly reflecting both *RA* and *RB* transcripts. The same blot was probed with a probe against *tubulin84B* (tub) to visualize mRNA levels.

(C) Real-time quantitative RT-PCR analysis of head mRNA using primers specific for the *RA*, *RC*, and *RC*+*RD* transcripts. Representative means of triplicate PCR reactions are shown. Each transcript level was normalized to the average of multiple independent control samples. The relative levels of all three transcripts were similar in wild-type controls (Ctl), a phenotypically wild-type $whir^1$ revertant line ($whir^{1REV}$), and the sedation-resistant $whir^{\Delta RC}$ strain. In the mutant $whir^1$ strain, *RA* levels were reduced greater than 40-fold, while *RC* levels were approximately 15-fold lower. Independent biological replicates showed essentially identical results.

(D) RT-PCR of the 5′ end of the *RC* transcript from $whir^{\Delta RC}$ showed a reduction in transcript size compared to the wild-type control (Ctl), as predicted by the 625 bp genomic deletion. M = molecular weight markers.

ethanol resistance observed with *whir* mutants. To test this, we compared the ethanol response of $whir^1$ flies to those carrying, in addition to $whir^1$, loss-of-function mutations in *Rho1* or the three genes encoding *Rac* homologs (*Rac1*, *Rac2*, and *Mtl*). We were unable to test the effect of mutations in *Cdc42* because this gene and *RhoGAP18B* are very closely linked on the X chromosome. Heterozygosity for two different loss-of-function alleles of *Rho1* strongly suppressed the $whir^1$ ethanol-resistance phenotype. Similar results were obtained upon reducing the dose of all three *Rac* homologs (Figure 3B), although this effect was sensitive to genetic background (see Experimental Procedures). These data suggest that *RhoGAP18B* functions through *Rho1* and/or possibly *Rac* to control ethanol-induced sedation.

To further investigate the involvement of the Rho-type GTPases in ethanol-induced behavior, we expressed transgenes encoding dominant-negative or constitutively active versions of these proteins (Luo et al., 1994) in flies using the GAL4/UAS system (Brand et al., 1994). Spatial restriction was achieved with the $whir^3$ enhancer-trap allele, which drives GAL4 expression in functionally relevant brain regions (see below); adult-onset expression of transgenes was achieved with the TARGET system (McGuire et al., 2004). Expression of constitutively active *Rac1* or *Rho1* transgenes, a condition expected to mimic *RhoGAP*

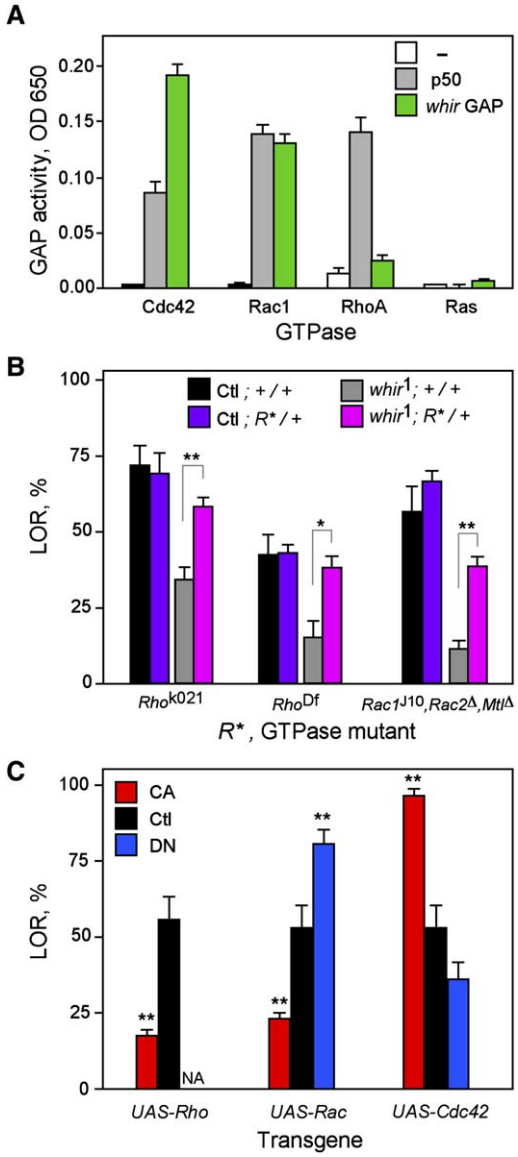

Figure 3. *RhoGAP18B* Encodes a Protein with GAP Activity In Vitro that May Regulate Rho and Rac In Vivo

(A) Amounts of inorganic phosphate produced by GTP hydrolysis. Purified fusion protein comprising the GAP domain from RhoGAP18B stimulated the GTPase activity of human Cdc42 and Rac1, but not RhoA (or the negative control Ras). The positive control p50 RhoGAP (aka ArhGAP1) stimulated GTPase activity of all three Rho-type GTPases, as previously described (Lancaster et al., 1994). White bars (–) denote the intrinsic GTPase activity upon addition of purified bacterial GST lysate lacking GAP.

(B) Heterozygosity for *Rho1* or all three *Rac* genes suppressed the ethanol resistance of *whir*^{1}. LOR was ascertained after 21 min of exposure to ethanol vapor (100/50 E/A). Strong loss-of-function alleles of Rho-type GTPases are denoted by *R**. The loss of one copy of *Rho1*, with *Df Rho1* or *Rho1*^{k021}, significantly suppressed the ethanol resistance of *whir*^{1} (**p < 0.01, t = 5.0; *p < 0.02, t = 3.3; t test, n = 4 experiments). The genetic interaction with *Rac* was less clear. Simultaneous loss of function of one copy each of the three *Rac* genes (*Rac1*, *Rac2*, and *Mtl*) showed suppression of the *whir*^{1} phenotype that was dependent on the mutant strain used (see Experimental Procedures).

loss of function, caused ethanol-resistance (Figure 3C). The opposite phenotype, enhanced ethanol sensitivity, was observed upon expression of the dominant-negative *Rac1* transgene (an equivalent *Rho* transgene was not available). In contrast, expression of the activated *Cdc42* transgene led to enhanced sensitivity, while expression of the dominant-negative transgene caused a tendency toward resistance. The fact that expression of overactive *Rho1* or *Rac1*, but not *Cdc42*, phenocopies the *whir* loss-of-function phenotype suggests that RhoGAP18B-PC acts through Rho1 and/or Rac GTPases, rather than Cdc42, to affect ethanol-induced sedation. These data are also in agreement with the genetic interactions observed between mutations in *whir* and *Rho/Rac* (Figure 3B).

white rabbit Is Expressed in the Adult Nervous System

Several strains carrying P element insertions in *RhoGAP18B* are GawB enhancer traps, in which the transcriptional activator GAL4 is expressed in cells likely to express endogenous *RhoGAP18B*. To examine this expression pattern, we generated flies that, in addition to a *whir* GawB insertion, carried a *UAS-GFP* reporter. GFP expression was analyzed in females heterozygous for the particular *whir* insertion (*whir/+;UAS-GFP*). Because these females showed normal ethanol sensitivity (Figure 1C), we expected them to recapitulate the expression of *RhoGAP18B* in wild-type flies. Expression of GAL4 was largely limited to the nervous system. In the adult brain, *whir*^{3}-driven expression was observed in the mushroom bodies, pars intercerebralis neurons, parts of the central complex (including the ellipsoid and fan-shaped bodies), some lateral neurons, a few olfactory projection neurons (arborizing on the DA1 and DM3 glomeruli), and unidentified neurons in the subesophageal ganglion (Figure 4A). Seven of eight GawB lines tested, including *whir*^{1} and *whir*^{3}, drove reporter-gene expression in essentially identical patterns (data not shown); *whir*^{3}, however, drove the highest level of expression and was therefore used for behavioral rescue experiments (see below).

To analyze the brain anatomy of *whir* mutant flies, we compared GFP reporter-gene expression in phenotypically

(C) Adult expression of dominant-negative (DN) or constitutively active (CA) forms of Rho-type GTPases affects ethanol sensitivity. Activated Rho1 led to resistance ($p < 0.01$, $t = 5.5$, $n = 5–8$), as did activated Rac1 ($p < 0.01$, $t = 3.7$, $n = 6–7$), while dominant-negative Rac1 resulted in ethanol sensitivity ($p < 0.01$, $t = 3.6$, $n = 6–7$). Cdc42 had the opposite effect, leading to sensitivity in the activated form ($p < 0.01$, $t = 6.0$, $n = 6–7$) and a tendency toward resistance in the dominant-negative form that was not statistically significant ($p = 0.07$, $t = 2.0$, $n = 6–8$). LOR after exposure to ethanol (120/30 E/A) was measured after 31 min for Rac and Cdc42 and after 21 min for Rho1 since the latter was in a different, and more sensitive, genetic background. Since developmental expression of the *UAS*-GTPase transgenes resulted in lethality, we expressed all transgenes in adults only utilizing the *whir*^{3}*-GAL4* driver and *Tub-GAL80*^{ts}. Specifically, experimental flies (*whir*^{3}*,GAL80*^{ts}*/+; UAS-GTPase/+*) and their controls were grown at 16°C and shifted to 29°C 2 days after eclosion. Behavioral testing was carried out 3 days later.

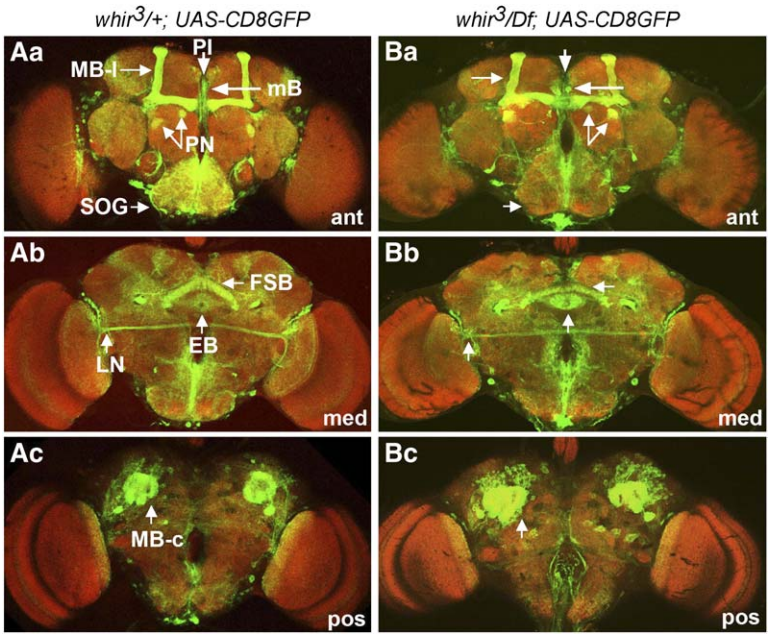

Figure 4. *whir*^{3} Drives GAL4 Expression in Specific Brain Regions

(A) Expression of GAL4 in wild-type females. Anterior (Aa), medial (Ab), and posterior (Ac) confocal stacks showing GFP expression (green) in the brains of *whir*^{3}*/+;UAS-mCD8-GFP* adult (phenotypically wild-type) females are shown. Brains were counterstained with the neuropil marker nc82 (red). Expression is observed in the mushroom body calyx (MB-c) and lobes (MB-l), antennal lobe projection neurons (PN; only dendritic arborizations in the antennal lobe are visible), lateral neurons (LN), ellipsoid body (EB), fan-shaped body (FSB), ventral subesophageal ganglion cells (SOG), and pars intercerebralis neurons (Pl).

(B) Confocal stacks obtained as in (A) from *whir*^{3}*/Df;UAS-mCD8-GFP* adult (phenotypically mutant) females. GFP expression is seen in all structures stained in the control females (A), indicating absence of gross anatomical defects in *whir*^{3} mutant flies. Staining in the SOG was generally higher in the wild-type flies.

wild-type (*whir*^{3}*/+;UAS-GFP*) and mutant (*whir*^{3}*/Df;UAS-GFP*) females (Figures 4A and 4B). All brain structures expressing GFP in wild-type flies were found to be present and apparently normal in mutant flies, although GFP expression in the subesophageal ganglion was in general higher in wild-type. The defective behavioral response to ethanol displayed by *whir* flies is therefore not caused by gross structural alterations of the nervous system, although we cannot rule out more subtle structural defects.

Expression of *RhoGAP18B-RC* Rescues *white rabbit*'s Ethanol Resistance

To study the functional relevance of the GAL4 expression pattern observed in *whir*^{3} flies and to analyze the role of the *RC* transcript in ethanol-induced sedation, we determined whether expression of a *UAS-RC* cDNA transgene in mutant *whir*^{3} males would rescue their behavioral phenotype. Indeed, males expressing *RC* in the *whir*^{3} pattern (*whir*^{3}*;UAS-RC*) showed nearly normal ethanol sedation compared to mutant *whir*^{3} males expressing an innocuous protein (*whir*^{3}*;UAS-GFP*) (Figures 5A and 5B). This behavioral rescue by *RC* expression was manifested as a reduction in the time required for 50% of the flies to reach sedation (ST_{50}; Figure 5B). Expression of the *UAS-RC* transgene in phenotypically wild-type, heterozygous females (*whir*^{3}*/+;UAS-RC)* had no effect (Figures 5A and 5B).

In addition to restoring nearly normal ethanol sedation to *whir*^{3} mutants, *RC* expression also increased the viability of the *whir*^{3} stock from 31% to 83% ($n > 150$ for each class, $p < 0.001$, chi-square test). Expression of *RC* in the fully viable *whir*^{1} mutant (*whir*^{1}*;UAS-RC*) similarly ameliorated the ethanol-resistance phenotype of this allele (Figure S4A). The phenotypic rescue was, however, incomplete, probably due to the fact that expression of GAL4 (and thus *RC*) in *whir*^{1} flies is lower than that achieved in *whir*^{3} flies (data not shown).

These data confirm that loss of the *RC* transcript is responsible for the ethanol resistance of *whir* mutant flies and show that the sites of *whir*^{3} enhancer-trap expression (Figure 4A) reflect regions where *RhoGAP18B-RC* functions to regulate sensitivity to the sedating effect of ethanol.

Expression of *RhoGAP18B-RC* in the Adult Fly Is Necessary and Sufficient for Normal Ethanol-Induced Sedation

To determine whether expression of *RhoGAP18B-RC* is required during development or in the adult fly to regulate ethanol-induced sedation, we used the TARGET system to regulate *RC* expression temporally (McGuire et al., 2004). Using this system, the activity of GAL4 is suppressed by a temperature-sensitive GAL80 at the permissive temperature (16°C), but not the restrictive (25° or 29°C) temperature.

We first asked whether *RhoGAP18B-RC* expression in adult flies is sufficient to confer normal ethanol-induced sedation to *whir*^{3} flies. We therefore raised the "experimental" flies (*whir*^{3}*,GAL80*^{ts}*;UAS-RC*) at 16°, transferred them to 29° for 3 days in adulthood (to allow *UAS-RC* expression), and then tested them for ethanol-induced sedation. In parallel, we raised a "mutant control" group (*whir*^{3}*,GAL80*^{ts}*;UAS-GFP*), which displays the *whir*^{3} mutant phenotype, and a "wild-type control" group (*whir*^{3}*/+, GAL80*^{ts}*;UAS-GFP*), which shows wild-type behavior. Adult-limited expression of *RC* in the "experimental" group resulted in a complete rescue of the *whir*^{3} phenotype; the "experimental" group showed a reduced ST_{50} compared to the "mutant control" group and an ST_{50}

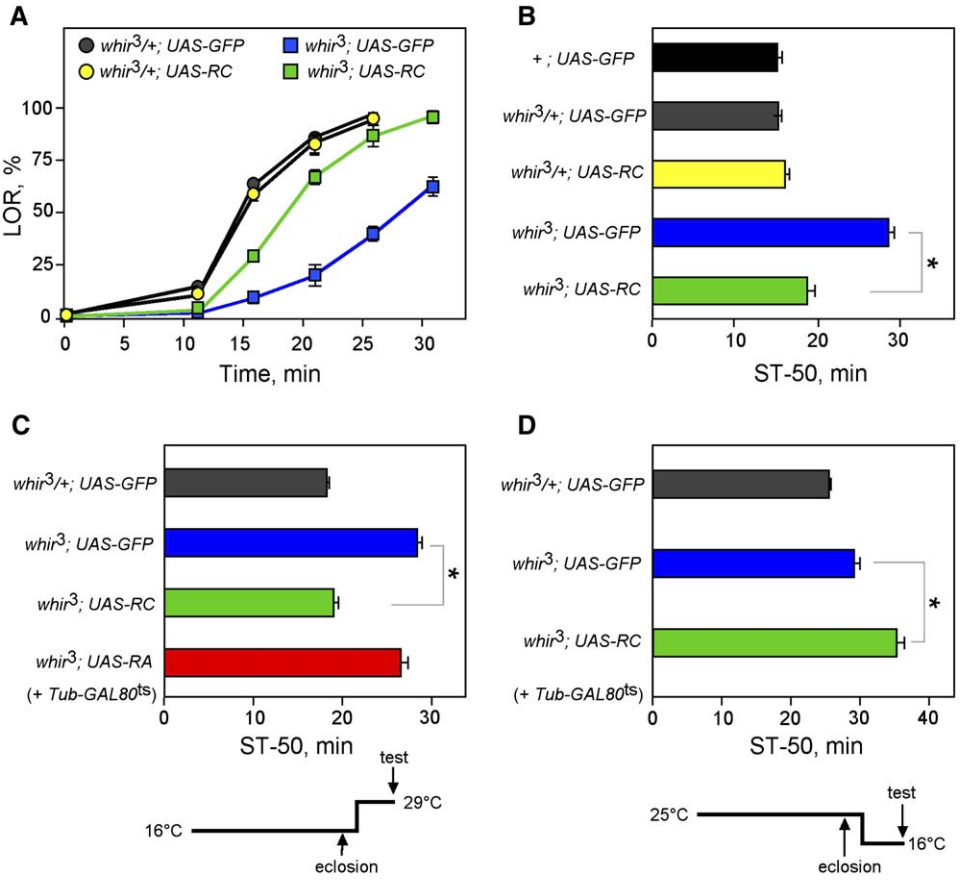

Figure 5. Adult Rescue of *whir*^{3} Sedation Resistance with an *RC* cDNA

(A) LOR as a function of ethanol exposure time (120/30 E/A). Expression of *RC* in *whir*^{3}/+ phenotypically wild-type females (*whir*^{3}/+;*UAS-RC*) did not alter the LOR profile compared to controls (*whir*^{3}/+;*UAS-GFP*). Expression of *RC* in *whir*^{3} mutant males (*whir*^{3};*UAS-RC*) shifted the LOR curve to earlier (more normal) times as compared to control *whir*^{3} males expressing an innocuous protein (*whir*^{3};*UAS-GFP*), indicating rescue of the *whir*^{3} sedation resistance. (n = 4 experiments).

(B) Median sedation time (ST_{50})—the time required for 10 of 20 ethanol-exposed flies to show LOR—of the genotypes shown in (A). This analysis shows significant rescue of the *whir*^{3} sedation defect by expression of the *RC* cDNA (*p < 0.001, t = 13, n = 6). Male control flies (+;*UAS-GFP*) were also included in this experiment and showed no difference from either of the *whir*^{3}/+ female control groups.

(C) ST_{50} of *whir*^{3} flies with adult-limited expression of *RC* or *RA* cDNAs. All four groups, each carrying the *Tub-GAL80*^{ts} transgene, were raised at 16°C until adulthood (to suppress *UAS* transgene expression) and then shifted to 29°C for 3 days (to allow *UAS* transgene expression) prior to behavioral testing. Adult *RC* expression completely rescued the sedation resistance of *whir*^{3} males: The ST_{50} of *whir*^{3},*GAL80*^{ts};*UAS-RC* "rescued" males is significantly lower than that of phenotypically mutant *whir*^{3},*GAL80*^{ts};*UAS-GFP* males (p < 0.001, t = 12, t test, n = 6 experiments) and indistinguishable from that of "wild-type" *whir*^{3}/+,*GAL80*^{ts};*UAS-GFP* heterozygous females. In contrast, adult expression of the *RA* cDNA had no significant effect: Compare mutant *whir*^{3},*GAL80*^{ts};*UAS-GFP* males with *RA*-expressing *whir*^{3},*GAL80*^{ts};*UAS-RA* males (p > 0.14, t = 1.8, t test, n = 3–6 experiments).

(D) ST_{50} of *whir*^{3},*GAL80*^{ts} mutant flies in which *RC* expression was limited to development. Flies were grown at 25°C (a temperature that produces sufficient transgene expression for phenotypic rescue; data not shown) until adulthood and then shifted to 16°C (to shut off transgene expression) for 3 days prior to behavioral testing. The sedation resistance of "mutant" *whir*^{3} flies (*whir*^{3},*GAL80*^{ts};*UAS-GFP*) was not significantly rescued by *RC* expression (in *whir*^{3},*GAL80*^{ts};*UAS-RC* flies), but was actually enhanced (*p < 0.001, t = 5.2, t test, n = 6). Note that the sedation resistance of *whir*^{3} mutant males compared to phenotypically wild-type females is less pronounced when the flies are kept at 16°C prior to testing (but still significant; p < 0.001, t = 5.1, t test, n = 6), indicating some temperature sensitivity of the *whir*^{3} allele. Mutant *whir*^{3} flies developmentally expressing *RA* could not be tested due to their high degree of lethality. Also, results from (C) and (D) cannot be compared directly to each other because the rearing temperature affects ethanol-induced sedation; flies raised at the lower temperature are more resistant (compare *whir*^{3},*GAL80*^{ts}/+;*UAS-GFP* in [C] and [D]).

indistinguishable from that of the "wild-type control" group (Figure 5C). Thus, *RhoGAP18B-RC* expression solely in adulthood is sufficient for flies to respond normally to the sedating effects of ethanol.

We next asked whether adult expression of *RhoGAP18-RC* is necessary to confer normal ethanol-induced sedation. Flies of the three groups described above were raised at 25°C (to allow expression of *UAS* transgenes) and then placed at 16°C for 3 days prior to behavioral testing. We observed that developmental expression of *RC* failed to rescue the *whir*^{3} phenotype (Figure 5D). Curiously, adult-specific shutdown of *RC* expression in the "experimental"

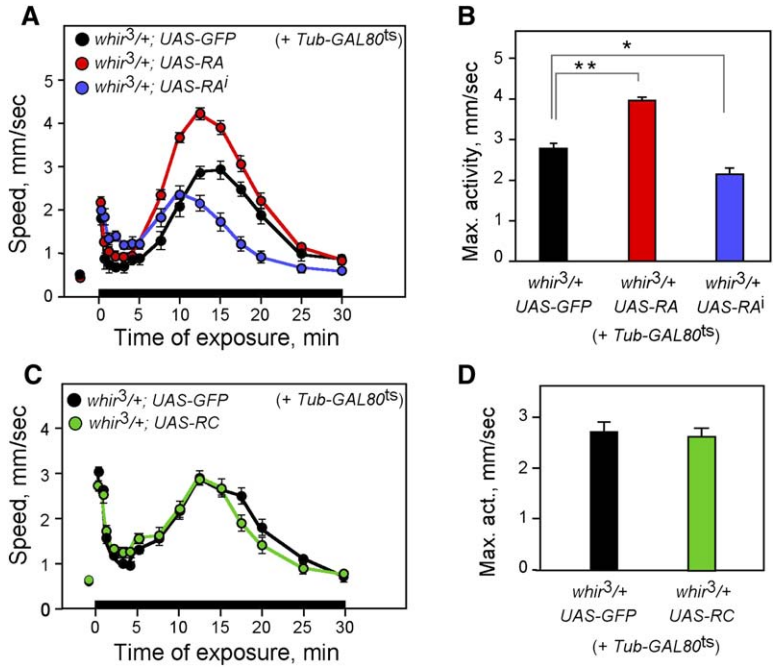

Figure 6. The *RA* Transcript Mediates Ethanol-Induced Hyperactivity

(A and B) Manipulations of *RA* transcript levels in the *whir*^{3} expression pattern affected ethanol-induced hyperactivity.

(A) Locomotion tracking profile of *whir*^{3}/+ females expressing an *RA* cDNA (*whir*^{3}/+; *UAS-RA*) or an *RA* RNAi transgene (*whir*^{3}/+; *UAS-RA*^{i}) in adults. All flies also carried the *Tub-GAL80*^{ts} transgene, and the temperature regimen was the same as described in Figure 5C (100/50 E/A). *RA* cDNA overexpression resulted in a pronounced hyperactivity, while *RA* RNAi expression led to blunted hyperactivity when compared to control flies expressing GFP (*whir*^{3}/+;*UAS-GFP*).

(B) The degree of hyperactivity was quantified by averaging the successive three time points with the highest speed of locomotion (**p < 0.001, t = 6.9; *p < 0.03, t = 2.4; t test; n = 13–15 experiments).

(C and D) Manipulations of *RC* transcript levels in the *whir*^{3} expression pattern do not affect ethanol-induced hyperactivity.

(C) Locomotion tracking profile of *whir*^{3}/+ females expressing *RC* cDNA (*whir*^{3}/+;*UAS-RC*) in adults.

(D) The profile and maximal activity of the flies in (C) is indistinguishable from those of control, GFP-expressing flies (*whir*^{3}/+;*UAS-GFP*). All flies also carried the *Tub-GAL80*^{ts} transgene, and the temperature regimen was the same as described in Figure 5C (100/50 E/A).

flies resulted in a worsening of the phenotype (i.e., a further increase in the ST_{50}) compared to the "mutant control" flies (Figure 5D). While we currently do not understand the reasons for the latter observation, our data show conclusively that *RhoGAP18B-RC* expression is not required during development but, rather, functions continuously in the adult fly to confer normal ethanol-induced sedation.

RhoGAP18B-RA Functions in Ethanol-Induced Hyperactivity

To determine whether the *RA* transcript—predicted to encode a RhoGAP that shares only the GTPase-activating domain with *RC*—is also involved in ethanol responsiveness, we asked whether expression of the *RA* cDNA would restore normal behavior to *whir* flies. As we did previously for *RC* (Figures 5A and 5B), we generated a *UAS-RA* transgene and asked whether it could rescue the sedation resistance of *whir*^{3} males. Curiously, mutant males expressing *RA* (*whir*^{3}*;UAS-RA*) showed a significantly reduced viability compared to *whir*^{3} males (17% and 31%, respectively; n > 150 for each class, p < 0.001, chi-square test); in addition, many *RA*-expressing escapers died early in adult life, precluding their behavioral testing. Thus, while *RC* expression rescued the reduced viability associated with *whir*^{3}, expression of *RA* enhanced it.

To restrict *RA* expression to adult flies and overcome the lethality associated with its developmental expression, we utilized the TARGET system as described above. Specifically, we raised experimental flies (*whir*^{3}*,GAL80*^{ts}; *UAS-RA*) at 16°C and then shifted them to 29°C for 3 days (to allow *RA* expression) prior to testing in the LOR assay. The flies were resistant to the sedating effects of ethanol (Figure 5C)—i.e., their ST_{50} was not significantly different from that of the "mutant control" group (*whir*^{3}*, GAL80*^{ts}*;UAS-GFP*) but was significantly increased compared to the "wild-type control" group (*whir*^{3}*/+,GAL80*^{ts}*; UAS-GFP*). Thus, adult expression of the *RA* transcript was unable to substitute for the loss of the *RC* transcript. These data are consistent with our observation that *whir* mutants that disrupt only the *RC* transcript (*whir*$^{\Delta RC}$) show the same ethanol-sedation defect as flies lacking *RC* and *RA*, such as *whir*^{1} and *whir*^{3} (Figure 1 and Figure 2).

We noticed, however, an increase in ethanol-induced hyperactivity upon adult expression of the *RA* transcript and therefore tested these flies in the locomotor tracking system, which allows a precise quantification of walking speed (Wolf et al., 2002). As shown in Figures 6A and 6B, adult overexpression of *RA* in wild-type flies (*whir*^{3}*/+, GAL80*^{ts}*;UAS-RA*) caused a significant increase in ethanol-induced hyperactivity compared to control flies overexpressing innocuous GFP (*whir*^{3}*/+,GAL80*^{ts}*;UAS-GFP*), quantified as the maximal hyperactivity achieved during the 30 min ethanol exposure (Figure 6B). In contrast, adult overexpression of the *RC* transcript (*whir*^{3}*/+,GAL80*^{ts}*;*

UAS-RC) did not change ethanol-induced hyperactivity (Figures 6C and 6D).

To further study the involvement of *RhoGAP18B-RA* in ethanol-induced hyperactivity, we generated an RNA interference construct, *UAS-RA*^{i}, that would specifically downregulate the levels of the *RA* transcript (Figure S6C). Adult downregulation of *RA* (in *whir*^{3}/+,*GAL80*^{ts};*UAS-RA*^{i} flies) led to a reduction in ethanol-induced hyperactivity (Figures 6A and 6B), the opposite effect of that observed upon adult overexpression of *RA*.

In summary, changes in *RA* expression, but not *RC* expression, in the adult affect the ability of the flies to respond to the stimulant effects of ethanol. Neither overexpression nor downregulation of *RA* caused changes in baseline locomotion or the extent of olfactory startle (observed in the first minute of ethanol exposure), implicating *RA* specifically in the sensitivity of flies to the locomotor-activating effects of ethanol. Because the behavioral effects observed upon altering *RA* transcript levels in all neurons (using the *Nrv2-GAL4* or *elav-GAL4* drivers; Figures S5 and S6) were essentially identical to those seen with more spatially restricted manipulations (using the *whir*^{3} driver), we conclude that *RA* functions in the neurons identified by the *whir*^{3} driver (Figure 4) to regulate the stimulant effects of ethanol.

DISCUSSION

Humans exhibit responses to ethanol that range from disinhibition and euphoria at low doses to motor uncoordination and stupor at higher doses. In animal models, including *Drosophila*, these distinct phases of ethanol intoxication can be modeled by measuring locomotor activity, which is enhanced by low-to-moderate ethanol doses (approximately 20 mM or 0.09%), and sedation, which occurs when internal ethanol levels approximate 45 mM (or 0.21%) (Scholz et al., 2000). While the behavioral transition from locomotor stimulation to sedation happens gradually as flies absorb increasing concentrations of ethanol with time of exposure, the genetic control of these behavioral responses can be distinct (Singh and Heberlein, 2000). Here we describe the phenotypic and molecular characterization of the *Drosophila white rabbit* (*whir*) gene, encoding several distinct RhoGAPs, which plays a critical role in the regulation of ethanol-induced behaviors. Curiously, different RhoGAP18B transcripts, *RA* and *RC*, regulate the stimulant and sedating effects of ethanol, respectively.

GTPases of the Rho superfamily and their RhoGAP regulators have been shown in a variety of systems to play crucial roles in nervous-system development. *whir* mutant flies, however, appear to have normal brain structure and integrity. Consistent with this structural data is our finding that *RhoGAP18B-RC* and *-RA* are not required during development to properly set up the neural circuits necessary for ethanol-induced behaviors but, rather, function in the adult nervous system to regulate behavior. A role for Rho-type GTPases in the mature nervous system is increasingly being recognized. For example, p190 RhoGAP has been implicated in fear conditioning in rats by regulating the activity of the downstream kinase ROCK in the amygdala (Lamprecht et al., 2002). In our behavioral paradigms, ethanol-induced hyperactivity and sedation develop over a period of 10–30 min of drug exposure. It is therefore possible that some form of neural plasticity—such as the development of acute functional tolerance (defined as tolerance that develops within a single ethanol exposure)—modulates the extent of the behavioral response. The ability of Rho GTPases (and the molecules, such as RhoGAPs, that affect their activity) to dynamically regulate the actin cytoskeleton and, consequently, the reorganization of axonal and dendritic branches (Bonhoeffer and Yuste, 2002; Luo, 2002) makes them ideally suited to regulate synaptic plasticity and behavior. Indeed, several Rho GTPase effectors have been implicated in learning and memory in rodent models (Meng et al., 2002; Dash et al., 2004) and mental retardation in humans (Ramakers, 2002; Calabrese et al., 2006), although the exact temporal requirements for these functions have not been established.

In the *Drosophila* nervous system, Rho1 has been implicated in axon stability, neuroblast proliferation, and dendrite morphogenesis (Lee et al., 2000; Billuart et al., 2001), while Rac has been shown to participate in axon growth, guidance, branching, and connectivity (Ng et al., 2002). The *whir* ethanol-sedation defect was substantially suppressed by reducing the gene dose of *Rho1* and possibly *Rac*. In addition, constitutively active Rho1 or Rac1 mirrored the *whir* loss-of-function phenotype, suggesting that RhoGAP18B acts through Rho1 and Rac in vivo to regulate ethanol sedation. In vitro, the RhoGAP18B GAP domain acted on mammalian Rac1 and Cdc42, but not RhoA, to enhance their GTPase activity. We were unable to test full-length RhoGAP18B-PC protein, as it was insoluble. Possibly, the PC protein could activate RhoA/Rho1's GTPase activity, as suggested by our genetic data. Alternatively, posttranslational regulation of RhoGAP18B proteins in vivo—e.g., by phosphorylation or phospholipid binding—could confer activity toward Rho1. Both such regulations have been shown to alter RhoGAP specificity (Minoshima et al., 2003; Ligeti et al., 2004). Curiously, a mutant RhoGAP18B-PC protein in which the catalytic arginine finger was substituted for alanine was still able to partially rescue the *whir*^{3} sedation defect (Figure S4B); however, a GAP domain containing this mutation also retained substantial GTPase activating capacity (Figure S4C). Thus, the exact biochemical properties and specificities of the different RhoGAP18B isoforms remain to be elucidated. How could these varied developmental and behavioral processes all require Rho1 and/or Rac? Specificity may be entailed through regulation of GTPase activity by the distinct RhoGAPs and/or their counteracting RhoGEFs. Consistent with the notion that GTPase regulation is important for specific GTPase effects is the finding that the *Drosophila* genome, while encoding only 7 Rho-type GTPases (Johndrow et al., 2004), encodes

approximately 21 RhoGAPs and 23 RhoGEF proteins (Schmidt and Hall, 2002; Bernards, 2003). Thus, while Rho1's function in axon stability is regulated by p190 RhoGAP (Lee et al., 2000), its role in ethanol-induced sedation appears to be regulated by RhoGAP18B.

Further evidence that differential regulation is key to in the multifaceted activities of Rho GTPases comes from our findings that the *RhoGAP18B-RC* and *-RA* transcripts are involved in distinct aspects of the behavioral response to ethanol. The *whir* locus encodes three RhoGAP proteins that differ substantially in their N termini while sharing the C-terminal GTPase-activating domain. We show that PA and PC have opposing effects on ethanol sensitivity and viability. Since all *whir* phenotypes are associated with *RhoGAP18B* function in *whir*^{3}*-GAL4* expressing cells, it is possible that the PA and PC proteins act in the same cells to regulate Rho GTPase activity in distinct ways. The divergent N termini of PA (176 amino acids) and PC (1025 amino acids) may be involved in differential activation of upstream pathways and/or competition for Rho1 regulation; they could also activate distinct GTPases and thereby regulate different downstream processes. Alternative splicing of RhoGAP transcripts is not uncommon (Richnau and Aspenstrom, 2001; Furuta et al., 2002). For example, the neuronally expressed rat chimerin 1 exists in two forms that differ in their expression pattern in the brain and their subcellular localization (Hall et al., 2001). Thus, alternative splicing of RhoGAPs in flies and mammalian systems appears to be a mechanism used to generate a large number of functionally distinct regulators of Rho-type GTPases, which in turn regulate diverse cellular processes.

How could changes in the function of small GTPases affect ethanol-induced behaviors? One possibility is that ethanol may directly affect the organization of the cytoskeleton. Indeed, recent evidence shows that ethanol has actin-remodeling activity in cultured cerebellar neurons, an activity that requires Eps8, a known regulator of actin dynamics (Di Fiore and Scita, 2002). Interestingly, *Eps8* knockout mice show behavioral resistance to ethanol (Offenhäuser et al., 2006 [this issue of *Cell*]). Alterations in the actin cytoskeleton can also affect the clustering of neurotransmitter receptors. For example, the destruction of actin fibers with latrunculin results in a rundown of GABA(A) receptor currents in cultured hippocampal neurons, and the concomitant loss of receptor clusters is enhanced by loss of Rac1 (Meyer et al., 2000). These data show that small Rho-type GTPases are involved in regulation of the GABA(A) receptor, which is known to mediate the sedating effects of ethanol. In addition to these postsynaptic effects, Rho GTPases have been shown to regulate neurotransmitter release in *C. elegans* (McMullan et al., 2006) and *Aplysia* (Humeau et al., 2002). It is therefore possible that, in conditions of impaired function of small GTPases (as in our *whir* mutants), abnormal actin dynamics lead to abnormal receptor clustering and/or neurotransmitter release and, consequently, altered behavior. The bidirectional regulation of ethanol sensitivity observed upon expression of overactive and inhibitory *Rac* transgenes argues against a nonspecific effect of manipulating GTPase activity and suggests that the cellular processes regulated by *Rac* and *Rho* in the adult fly play a relatively direct role in the behavioral phenotype. A further argument for specificity derives from the finding that manipulations of *Rho* and *Rac* activity lead to phenotypes opposite to those observed with equivalent alterations in *Cdc42* function. Regardless of the exact mechanisms underlying these distinct effects, our data clearly show that Rho-type GTPases are intimately involved in the regulation of behavioral responses to ethanol exposure, thus implicating actin dynamics in the process.

EXPERIMENTAL PROCEDURES

Fly Stocks and Genetics

Flies were grown and kept on standard cornmeal/agar medium at 25°C. The genetic screen was carried out in the *w* Berlin wild-type background with the P{GawB} element (Brand et al., 1994) and will be described in detail elsewhere (A.R. and U.H., unpublished data). We isolated two alleles (*whir*^{2} and *whir*^{6}) due to their resistance to ethanol-induced sedation in the locomotor tracking system, three additional alleles due to their resistance to nicotine (*whir*^{1}, *whir*^{4}, and *whir*^{5}), and three alleles due to their resistance to cocaine (*EP1326*, *EP1439*, and *EP1621*; not characterized further). All alleles showed strong resistance to ethanol-induced sedation, and the P element insertions cluster near the *whir*^{1} and *whir*^{2} insertion sites. Additional alleles were obtained from the Japanese NP consortium (GETDB lines, including *whir*^{3} = NP1514). All insertions were outcrossed to both *w* Berlin and *w* Canton-S strains for five generations to remove unlinked modifiers and homogenize the genetic background. Other than Figure 1A and Figure 3 (see below), all experiments were conducted in the *w* Berlin background. Precise excisions were carried out in dysgenic females, and imprecise excisions in dysgenic males, utilizing the *{Δ2-3} Dr* jump-starter chromosome (Robertson et al., 1988). All transgenes were injected into *w* Berlin flies. The following mutant GTPase alleles were obtained from the Bloomington Drosophila Stock Center: *Df(2R)Jp6* (*Rho1*$^{-}$), *Rho1*^{k02107b}, *Rac2*^{Δ} *Mtl*^{Δ} *Rac1*^{J10}, and *Rac2*^{Δ} *Mtl*^{Δ} *Rac1*^{J11}. In all experiments, the genetic background in experimental and control flies was essentially identical (*w* Berlin), with the exception of experiments involving *Rho/Rac/Cdc42* strains, where the experimental and control flies were in the same hybrid genetic background (Canton-S/unknown or Berlin/unknown; the only difference between experimental and control flies being the chromosome carrying the GTPase mutation). The *Rac2*^{Δ} *Mtl*^{Δ} *Rac1*^{J10} stock consistently suppressed the *whir*^{1} sedation defect (Figure 3B), while the *Rac2*^{Δ} *Mtl*^{Δ} *Rac1*^{J11} stock suppressed sedation in some experiments, but not in others. In addition, the latter stock showed sensitivity in a wild-type background (which may be expected from loss of *Rac* function), thus precluding any conclusions regarding specific genetic interactions between *Rac* and *whir*.

Behavior

Locomotion video tracking was performed as described (Wolf et al., 2002). Twenty flies per tube were exposed to ethanol vapor. The LOR of ethanol-exposed flies was measured during the ethanol exposure every 5 min by lightly tapping the tube and then counting the flies unable to right themselves. The experimenter was blinded to the flies' genotype in all experiments. The time to 50% LOR was calculated for each exposure tube by linear interpolation of the two time points around the median and then averaged over the number of tubes (experiments). Hyperactivity was quantified by averaging the three successive time points with the highest locomotion speed for each

experiment and then averaging that number over replicate experiments. The data shown in most behavior figures were collected from assays performed on a single day, to eliminate day-to-day variability. However, all experiments were repeated on multiple days, with essentially identical results. Experiments shown in Figures 6A and 6B were pooled from 3 days due to low number of flies and exposure tubes on each day. Statistical analyses were performed in STATISTICA (StatSoft, Tulsa, OK, USA), and the specific tests used are indicated in the text and/or figure legends.

Immunohistochemistry

Immunocytochemistry was performed essentially as described (Marin et al., 2002). GFP fluorescence was visualized directly, and the neuropil was stained with the nc82 monoclonal antibody (Laissue et al., 1999).

Molecular Biology

Standard molecular techniques were utilized for the generation of constructs and RNA analysis (Sambrook et al., 2001). The P{GawB} element insertion sites on the X chromosome were as follows: *whir*^{1}, 18884637; *whir*^{2}, 18884596; *whir*^{3}, 18877652. All ESTs for *CG7502* (http://flybase.bio.indiana.edu/) represent the *RD* transcript that starts at position 18884414. No ESTs have been described for the *RC* transcript. We found biological evidence for *RC* on northern blots and by RT-PCR (data not shown; see also Figure 2). In addition, the *RC* ORF is conserved in multiple *Drosophila* species, including *D. pseudoobscura*, *D. yakuba*, and others. RT-PCR was performed on random-primed cDNA fragments from head RNA. Quantitative RT-PCR was performed as described in Tsai et al. (2004) using the primers listed in Supplemental Experimental Procedures.

Transgene Generation

UAS-RA cDNA was generated by cloning full-length EST LD25711 into pUAST, and *UAS-RC* cDNA was generated by cloning *RD* full-length *RE42510* into pUAST and adding a SacI/KpnI genomic PCR fragment amplified with 5′-CGAGAACTAAGGTCGTTTTTGGGGTG-3′ and 5′-GTGAGGGGCGCTCTGCCTCG-3′. The RNAi construct targeting *RA* (*UAS-RA^{i}*) was designed against the first exon of *RA* (that is shared with *RB*). A PCR fragment was amplified with primers 5′-GGTCTAGT CCATTCCATGCC-3′ and 5′-TGGGTTTCCACACTCGCTGCAGG-3′ and cloned into pWIZ (Lee and Carthew, 2003).

Biochemistry

A fragment of RhoGAP18B-PA from amino acids 222–466 was cloned into the GST expression vector pDest15 via pENTR (Invitrogen, Carlsbad, CA, USA). This fragment contains the GAP domain (291–456) and corresponds to the fragment of p50 RhoGAP that was expressed to obtain X-ray structure data (Barrett et al., 1997). 0.4 μg of purified GST-GAP domain was added to human GTPases (BK105 kit, Cytoskeleton, Denver), and inorganic phosphate production was measured after 10 min according to the manufacturer's specifications.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, and six figures and can be found with this article online at http://www.cell.com/cgi/content/full/127/1/199/DC1/.

ACKNOWLEDGMENTS

We thank members of the Heberlein laboratory for discussions and critical reading of the manuscript, especially Aylin Rodan, Doug Guarnieri, Rachael French, and Mark Eddison. We also thank Kevin Lease and Jay Hirsh for communicating results prior to publication; the Bloomington Drosophila Stock Center, Liqun Luo, and the Japanese NP consortium for fly strains; and Alois Hofbauer for nc82 antibody. Susan Parkhurst and Jörg Grosshans kindly provided expression vectors. The manuscript benefited greatly from comments by Justin Blau and Larry Tecott. This research was supported by the Swiss Society for Biomedical Stipends (A.R.) and NIH grants AA10035, AA13105, and DA14809 (U.H.).

Received: May 26, 2005
Revised: May 19, 2006
Accepted: September 4, 2006
Published: October 5, 2006

REFERENCES

Barrett, T., Xiao, B., Dodson, E.J., Dodson, G., Ludbrook, S.B., Nurmahomed, K., Gamblin, S.J., Musacchio, A., Smerdon, S.J., and Eccleston, J.F. (1997). The structure of the GTPase-activating domain from p50rhoGAP. Nature *385*, 458–461.

Bernards, A. (2003). GAPs galore! A survey of putative Ras superfamily GTPase activating proteins in man and Drosophila. Biochim. Biophys. Acta *1603*, 47–82.

Billuart, P., Winter, C.G., Maresh, A., Zhao, X., and Luo, L. (2001). Regulating axon branch stability: the role of p190 RhoGAP in repressing a retraction signaling pathway. Cell *107*, 195–207.

Bonhoeffer, T., and Yuste, R. (2002). Spine motility. Phenomenology, mechanisms, and function. Neuron *35*, 1019–1027.

Brand, A.H., Manoukian, A.S., and Perrimon, N. (1994). Ectopic expression in Drosophila. Methods Cell Biol. *44*, 635–654.

Calabrese, B., Wilson, M.S., and Halpain, S. (2006). Development and regulation of dendritic spine synapses. Physiology (Bethesda) *21*, 38–47.

Carlisle, H.J., and Kennedy, M.B. (2005). Spine architecture and synaptic plasticity. Trends Neurosci. *28*, 182–187.

Cloninger, C.R. (1987). Neurogenetic adaptive mechanisms in alcoholism. Science *236*, 410–416.

Dash, P.K., Orsi, S.A., Moody, M., and Moore, A.N. (2004). A role for hippocampal Rho-ROCK pathway in long-term spatial memory. Biochem. Biophys. Res. Commun. *322*, 893–898.

Devor, E.J., and Cloninger, C.R. (1989). Genetics of alcoholism. Annu. Rev. Genet. *23*, 19–36.

Di Fiore, P.P., and Scita, G. (2002). Eps8 in the midst of GTPases. Int. J. Biochem. Cell Biol. *34*, 1178–1183.

Enoch, M.A., and Goldman, D. (1999). Genetics of alcoholism and substance abuse. Psychiatr. Clin. North Am. *22*, 289–299.

Etienne-Manneville, S., and Hall, A. (2002). Rho GTPases in cell biology. Nature *420*, 629–635.

Furuta, B., Harada, A., Kobayashi, Y., Takeuchi, K., Kobayashi, T., and Umeda, M. (2002). Identification and functional characterization of nadrin variants, a novel family of GTPase activating protein for rho GTPases. J. Neurochem. *82*, 1018–1028.

Guarnieri, D.J., and Heberlein, U. (2003). Drosophila melanogaster, a genetic model system for alcohol research. Int. Rev. Neurobiol. *54*, 199–228.

Hakeda-Suzuki, S., Ng, J., Tzu, J., Dietzl, G., Sun, Y., Harms, M., Nardine, T., Luo, L., and Dickson, B.J. (2002). Rac function and regulation during Drosophila development. Nature *416*, 438–442.

Hall, C., Michael, G.J., Cann, N., Ferrari, G., Teo, M., Jacobs, T., Monfries, C., and Lim, L. (2001). alpha2-chimaerin, a Cdc42/Rac1 regulator, is selectively expressed in the rat embryonic nervous system and is involved in neuritogenesis in N1E-115 neuroblastoma cells. J. Neurosci. *21*, 5191–5202.

Hodge, C.W., Mehmert, K.K., Kelley, S.P., McMahon, T., Haywood, A., Olive, M.F., Wang, D., Sanchez-Perez, A.M., and Messing, R.O. (1999). Supersensitivity to allosteric GABA(A) receptor modulators and alcohol in mice lacking PKCepsilon. Nat. Neurosci. *2*, 997–1002.

Humeau, Y., Popoff, M.R., Kojima, H., Doussau, F., and Poulain, B. (2002). Rac GTPase plays an essential role in exocytosis by controlling the fusion competence of release sites. J. Neurosci. *22*, 7968–7981.

Johndrow, J.E., Magie, C.R., and Parkhurst, S.M. (2004). Rho GTPase function in flies: insights from a developmental and organismal perspective. Biochem. Cell Biol. *82*, 643–657.

Laissue, P.P., Reiter, C., Hiesinger, P.R., Halter, S., Fischbach, K.F., and Stocker, R.F. (1999). Three-dimensional reconstruction of the antennal lobe in Drosophila melanogaster. J. Comp. Neurol. *405*, 543–552.

Lamprecht, R., Farb, C.R., and LeDoux, J.E. (2002). Fear memory formation involves p190 RhoGAP and ROCK proteins through a GRB2-mediated complex. Neuron *36*, 727–738.

Lancaster, C.A., Taylor-Harris, P.M., Self, A.J., Brill, S., van Erp, H.E., and Hall, A. (1994). Characterization of rhoGAP. A GTPase-activating protein for rho-related small GTPases. J. Biol. Chem. *269*, 1137–1142.

Lee, A., Li, W., Xu, K., Bogert, B.A., Su, K., and Gao, F.B. (2003). Control of dendritic development by the Drosophila fragile X-related gene involves the small GTPase Rac1. Development *130*, 5543–5552.

Lee, T., Winter, C., Marticke, S.S., Lee, A., and Luo, L. (2000). Essential roles of Drosophila RhoA in the regulation of neuroblast proliferation and dendritic but not axonal morphogenesis. Neuron *25*, 307–316.

Lee, Y.S., and Carthew, R.W. (2003). Making a better RNAi vector for Drosophila: use of intron spacers. Methods *30*, 322–329.

Ligeti, E., Dagher, M.C., Hernandez, S.E., Koleske, A.J., and Settleman, J. (2004). Phospholipids can switch the GTPase substrate preference of a GTPase-activating protein. J. Biol. Chem. *279*, 5055–5058.

Lundstrom, A., Gallio, M., Englund, C., Steneberg, P., Hemphala, J., Aspenstrom, P., Keleman, K., Falileeva, L., Dickson, B.J., and Samakovlis, C. (2004). Vilse, a conserved Rac/Cdc42 GAP mediating Robo repulsion in tracheal cells and axons. Genes Dev. *18*, 2161–2171.

Luo, L. (2002). Actin cytoskeleton regulation in neuronal morphogenesis and structural plasticity. Annu. Rev. Cell Dev. Biol. *18*, 601–635.

Luo, L., Liao, Y.J., Jan, L.Y., and Jan, Y.N. (1994). Distinct morphogenetic functions of similar small GTPases: Drosophila Drac1 is involved in axonal outgrowth and myoblast fusion. Genes Dev. *8*, 1787–1802.

Maas, J.W., Jr., Vogt, S.K., Chan, G.C., Pineda, V.V., Storm, D.R., and Muglia, L.J. (2005). Calcium-stimulated adenylyl cyclases are critical modulators of neuronal ethanol sensitivity. J. Neurosci. *25*, 4118–4126.

Marin, E.C., Jefferis, G.S., Komiyama, T., Zhu, H., and Luo, L. (2002). Representation of the glomerular olfactory map in the Drosophila brain. Cell *109*, 243–255.

McGuire, S.E., Mao, Z., and Davis, R.L. (2004). Spatiotemporal gene expression targeting with the TARGET and gene-switch systems in Drosophila. Sci. STKE *2004*, pl6.

McMullan, R., Hiley, E., Morrison, P., and Nurrish, S.J. (2006). Rho is a presynaptic activator of neurotransmitter release at pre-existing synapses in C. elegans. Genes Dev. *20*, 65–76.

Meng, Y., Zhang, Y., Tregoubov, V., Janus, C., Cruz, L., Jackson, M., Lu, W.Y., MacDonald, J.F., Wang, J.Y., Falls, D.L., and Jia, Z. (2002). Abnormal spine morphology and enhanced LTP in LIMK-1 knockout mice. Neuron *35*, 121–133.

Meyer, D.K., Olenik, C., Hofmann, F., Barth, H., Leemhuis, J., Brunig, I., Aktories, K., and Norenberg, W. (2000). Regulation of somatodendritic GABAA receptor channels in rat hippocampal neurons: evidence for a role of the small GTPase Rac1. J. Neurosci. *20*, 6743–6751.

Meyer, G., and Feldman, E.L. (2002). Signaling mechanisms that regulate actin-based motility processes in the nervous system. J. Neurochem. *83*, 490–503.

Minoshima, Y., Kawashima, T., Hirose, K., Tonozuka, Y., Kawajiri, A., Bao, Y.C., Deng, X., Tatsuka, M., Narumiya, S., May, W.S., Jr., et al. (2003). Phosphorylation by aurora B converts MgcRacGAP to a RhoGAP during cytokinesis. Dev. Cell *4*, 549–560.

Moore, M.S., DeZazzo, J., Luk, A.Y., Tully, T., Singh, S.M., and Heberlein, U. (1998). Ethanol Intoxication in *Drosophila*: Genetic and Pharmacological Evidence for Regulation by the cAMP Signaling Pathway. Cell *93*, 997–1007.

Ng, J., Nardine, T., Harmns, M., Tzu, J., Goldstein, A., Sun, Y., Dietzl, G., Dickson, B.J., and Luo, L. (2002). Rac GTPases control axon growth, guidance and branching. Nature *416*, 442–447.

Offenhäuser, N., Castelletti, D., Mapelli, L., Soppo, B.E., Regondi, M.C., Rossi, P., D'Angelo, E., Frassoni, C., Amadeo, A., Tocchetti, A., et al. (2006). Increased ethanol resistance and consumption in *Eps8* knockout mice correlates with altered actin dynamics. Cell *127*, this issue, 213–226.

Park, S.K., Sedore, S.A., Cronmiller, C., and Hirsh, J. (2000). PKA-RII-deficient *Drosophila* are viable but show developmental, circadian and drug response phenotypes. J. Biol. Chem. *275*, 20588–20596.

Parr, J., Large, A., Wang, X., Fowler, S.C., Ratzlaff, K.L., and Ruden, D.M. (2001). The inebri-actometer: a device for measuring the locomotor activity of Drosophila exposed to ethanol vapor. J. Neurosci. Methods *107*, 93–99.

Ramakers, G.J. (2002). Rho proteins, mental retardation and the cellular basis of cognition. Trends Neurosci. *25*, 191–199.

Richnau, N., and Aspenstrom, P. (2001). Rich, a rho GTPase-activating protein domain-containing protein involved in signaling by Cdc42 and Rac1. J. Biol. Chem. *276*, 35060–35070.

Robertson, H.M., Preston, C.R., Phillis, R.W., Johnson-Schlitz, D.M., Benz, W.K., and Engels, W.R. (1988). A stable genomic source of P element transposase in Drosophila melanogaster. Genetics *118*, 461–470.

Sambrook, J., MacCallum, P., and Russell, D. (2001). Molecular Cloning: A Laboratory Manual, Third Edition (Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press).

Schmidt, A., and Hall, A. (2002). Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. Genes Dev. *16*, 1587–1609.

Scholz, H., Ramond, J., Singh, C.M., and Heberlein, U. (2000). Functional ethanol tolerance in *Drosophila*. Neuron *28*, 261–271.

Schuckit, M.A. (2000). Genetics of the risk for alcoholism. Am. J. Addict. *9*, 103–112.

Schuckit, M.A., Smith, T.L., and Kalmijn, J. (2004). The search for genes contributing to the low level of response to alcohol: patterns of findings across studies. Alcohol. Clin. Exp. Res. *28*, 1449–1458.

Scott, E.K., Reuter, J.E., and Luo, L. (2003). Small GTPase Cdc42 is required for multiple aspects of dendritic morphogenesis. J. Neurosci. *23*, 3118–3123.

Sin, W.C., Haas, K., Ruthazer, E.S., and Cline, H.T. (2002). Dendrite growth increased by visual activity requires NMDA receptor and Rho GTPases. Nature *419*, 475–480.

Singh, C.M., and Heberlein, U. (2000). Genetic control of acute ethanol-induced behaviors in Drosophila. Alcohol. Clin. Exp. Res. *24*, 1127–1136.

Thiele, T.E., Marsh, D.J., Ste. Marie, L., Bernstein, I.L., and Palmiter, R.D. (1998). Ethanol consumption and resistance are inversely related to neuropeptide Y levels. Nature *396*, 366–369.

Thiele, T.E., Willis, B., Stadler, J., Reynolds, J.G., Bernstein, I.L., and McKnight, G.S. (2000). High ethanol consumption and low sensitivity to ethanol-induced sedation in protein kinase A-mutant mice. J. Neurosci. (Online) *20*, RC75.

Thiele, T.E., Sparta, D.R., Hayes, D.M., and Fee, J.R. (2004). A role for neuropeptide Y in neurobiological responses to ethanol and drugs of abuse. Neuropeptides *38*, 235–243.

Tsai, L.T., Bainton, R.J., Blau, J., and Heberlein, U. (2004). Lmo mutants reveal a novel role for circadian pacemaker neurons in cocaine-induced behaviors. PLoS Biol. *2*, e408.

Wen, T., Parrish, C.A., Xu, D., Wu, Q., and Shen, P. (2005). Drosophila neuropeptide F and its receptor, NPFR1, define a signaling pathway that acutely modulates alcohol sensitivity. Proc. Natl. Acad. Sci. USA *102*, 2141–2146.

Wolf, F.W., Rodan, A.R., Tsai, L.T., and Heberlein, U. (2002). High-resolution analysis of ethanol-induced locomotor stimulation in Drosophila. J. Neurosci. *22*, 11035–11044.

Accession Numbers

The GenBank accession numbers for *RhoGAP18B-RB*, *-RD*, and *-RC* are DQ486141–DQ486143.