Published in final edited form as:

Genes Brain Behav. 2008 August ; 7(6): 669–676. doi:10.1111/j.1601-183X.2008.00404.x.

Loss of RAB-3/A in *C. elegans* and the mouse affects behavioral response to ethanol

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Abstract

The mechanisms by which ethanol induces changes in behavior are not well understood. Here we show that *C. elegans* loss-of-function mutations in the synaptic vesicle-associated RAB-3 protein and its GTP exchange factor AEX-3 confer resistance to the acute locomotor effects of ethanol. Similarly, mice lacking one or both copies of *Rab3A* are resistant to the ataxic and sedative effects of ethanol, and *Rab3A* haploinsufficiency increases voluntary ethanol consumption. These data suggest a conserved role of RAB-3/RAB3A-regulated neurotransmitter release in ethanol-related behaviors.

Keywords

RAB-3/RAB3A; AEX-3; presynaptic; neurotransmitter release; ethanol resistance; ethanol consumption

INTRODUCTION

Ethanol has similar intoxicating effects in vertebrate and invertebrate systems. In humans, ethanol causes loss of both physical coordination and social inhibition at low doses, and incoherence and sedation at high doses. Similarly, in *C. elegans* and *Drosophila*, the same tissue concentrations of ethanol cause loss of coordination and sedation (Davies *et al.*, 2003; Singh and Heberlein, 2000). Furthermore, mutational analysis has identified several genes as important for regulating the behavioral response to ethanol in multiple organisms. These include components of the cyclic AMP/PKA signaling pathway (Ferraro *et al.*, 2006; Moore *et al.*, 1998; Pandey *et al.*, 2004; Tanaka *et al.*, 2004; Thiele *et al.*, 2000; Wand *et al.*, 2001; Yang *et al.*, 2003) and BK-type potassium channel (Cowmeadow *et al.*, 2005, 2006; Davies *et al.*, 2003). Taken together, these data suggest that the mechanisms of ethanol's action may be conserved across species.

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Although some molecular targets of ethanol have been identified, the mechanisms contributing to the behavioral effects of ethanol are poorly understood. Considerable evidence has established a role for ethanol at the level of the synapse. To date, biochemical and genetic studies have primarily demonstrated an effect of ethanol on postsynaptic targets, including alterations in glutamatergic and GABAergic receptors, scaffolding proteins, and intracellular signal transduction pathways (for review see Chandler, 2003). Evidence for presynaptic effects of ethanol has been less forthcoming, although Nie *et al.* (1997) have reported that blocking presynaptic metabotropic GABA_B receptors in the rat brain enhances evoked transmitter release upon acute ethanol administration. Consistent with these observations, acute ethanol treatment increases GABA release in mouse VTA neurons and chronic ethanol treatment increases GABA release in rat central amygdala (Melis *et al.*, 2002; Roberto *et al.*, 2004). In *Drosophila*, RNA interference of dGABA_BR1 and treatment with the GABA_B antagonist CGP 54626 attenuates ethanol-induced motor impairment (Dzitoyeva *et al.*, 2003). However, it is unclear whether these effects are regulated pre- or postsynaptically.

RAB-3/RAB3A is a small G-protein expressed almost exclusively in neurons (Fischer von Mollard *et al.*, 1990) and interacts directly with synaptic vesicles to regulate their release (Geppert *et al.*, 1994, Iwasaki *et al.*, 1997, Nonet *et al.*, 1997). To determine if genetic disruption of this presynaptic protein alters the behavioral response to ethanol, we have analyzed *rab-3/Rab3A* loss-of-function mutants in *C. elegans* and the mouse for ethanol-related behaviors.

METHODS

C. elegans methods

Nematode culture and strains—Nematodes were cultured using standard methods (Brenner, 1974). The strains used in this study were: N2 var. Bristol, *rab-3(js49)*, *rab-3(y250)*, *aex-3(y255)*, *aex-3(sa5)* and *aex-3(n2166)*, and *cab-1(tg46)*. The *rab-3*, *aex-3* and *cab-1* mutants are all strong loss-of-function alleles for the respective genes. In all analyses, first day adult animals of each strain were used.

Dispersal assays—Dispersal assays were performed as described (Crowder *et al.*, 1996) with modifications: Standard large (10 cm) NGM plates were seeded with a ring of *E. coli* bacteria (OP50) near the edge of the plate which was allowed to grow for 2 hours at 37°C. A 2 mm diameter copper wire was melted into the surface of the plate to divide the plate into two semicircles. A representative plate was melted to determine the volume, and 100% ethanol was added to test plates to an appropriate final concentration (0mM, 200mM or 400mM). 100% ethanol will kill bacteria and render it unattractive to the worms, so care was taken to place the ethanol away from the food. The plate was sealed with Parafilm and the ethanol was allowed to equilibrate in the agar overnight at room temperature. On the day of the assay, approximately 100 animals of each strain were incubated in M9 buffer and ethanol [(22mM KH₂PO₄, 22mM Na₂HPO₄, 85mM NaCl, 1mM MgSO₄) and the appropriate concentration of ethanol (0mM, 200mM or 400mM)] for 20 minutes. Worms were spun down, and the pellet was pipetted to a central spot near the dividing bar, and the worms were allowed to move toward the food. Mutant and control strains were treated in parallel and placed on the plate in opposite semicircles at the same time. Worms were counted at 10-minute intervals. A dispersal index (DI) was calculated: DI= worms in food/total worms in assay. In this and the analysis of locomotion experiments, we have chosen to treat the animals with an exogenous dose of 400mM ethanol for three reasons. First, treatment with 400mM ethanol generates an internal ethanol concentration that is consistent with a concentration that would intoxicate humans. Second, 400mM is sufficient to induce

significant reversible behavioral inhibition in *C. elegans*. Third, we have used a 400mM ethanol dose in our previous acute locomotion studies (Davies *et al.* 2003) and have maintained this consistency to allow a comparison of results across experiments.

Analysis of locomotion—The effects of ethanol on *C. elegans* locomotion rate were assayed as described (Davies *et al.*, 2003). NGM plates were used without a bacterial lawn. The assay plates were dried at 37°C for 2 hours, copper rings were melted into the surface and ethanol was added to a final concentration of 0mM or 400mM. The plates were sealed with Parafilm and left for 2 hours at room temperature. Ten animals were moved to a plate without bacteria for 30 minutes prior to the assay then placed on the assay plate and their movement recorded at 20 minutes. Subjects were video recorded for 2 minutes using a CCD camera (1 frame/second). The time-lapse recordings were analyzed using DIAS software (Solltech, Inc., Atlanta, GA, US). To minimize the effects of differences in the basal speed of each strain we calculated a relative speed for each strain (treated average speed/untreated average speed $\times 100$, $n = 3$). To eliminate possible plate-to-plate variation in treatment conditions, the relative locomotion of each mutant strain was compared to the locomotion of controls treated on the same ethanol (or no ethanol) plates as the experimental group.

Internal ethanol concentration measurements—Internal ethanol concentrations following exposure to ethanol were determined as follows. In order to determine the internal concentration of ethanol during the period quantified during the analysis of locomotion, ethanol treatment plates were prepared exactly as described for the analysis of locomotion. 300–600 worms were placed on dried (37°C for 2 hours) plates without bacteria that had ethanol added to a final concentration of 0mM or 400mM. The animals were left on the plates for 20 minutes before they were washed from the plate with ice-cold dH₂O. The animals were briefly centrifuged at 4°C to form a pellet and the supernatant was removed. The volume of the worm-containing pellet was determined and ice-cold dH₂O was added to a final volume of 30 μ l. The worms were frozen at –85°C for 30 min before homogenization on ice. The concentration of ethanol in the homogenate was determined according to the manufacturer's instructions using an Alcohol Reagent Kit (Pointe Scientific, Canton, MI, US). Strain comparisons were performed using a two-tailed t-test.

Mouse methods

Subjects—*Rab3A^{tm1Sud}* mice (Jackson Laboratories, Bar Harbor, ME, US) maintained on a mixed C57BL/6J X 129/SvJ background were backcrossed to wild-type C57BL/6J mice for 6 generations prior to intercrossing of *Rab3A^{tm1Sud}/+* heterozygous mice to generate progeny for behavioral testing. Male mice were housed 3–5 per cage and were 2–4 months of age at onset of behavioral testing and 4–6 months of age upon completion of testing. All animal protocols were approved by the EGCRC institutional animal care and use committee.

Behavioral Testing—Each mouse was subjected to the following series of behavioral assays: week 1: stationary dowel test; weeks 2–4: loss-of righting reflex assay testing response to single 3.2, 3.6, and 4.0 doses EtOH presented weekly in pseudorandom order; weeks 5–8: 2-bottle choice test for EtOH consumption/preference; week 9: 2-bottle choice test for saccharin and quinine taste preference. A separate, drug-naïve cohort of subjects was used to assess ethanol metabolism and clearance.

Stationary dowel assay—A horizontal wooden dowel (1.5 cm diameter, 30 cm long) was suspended between two Plexiglas walls 50 cm above a cushioned surface. Mice were trained to remain on the stationary dowel for 5 min. Each mouse was then given an IP injection of 1.5 g/kg of ethanol (10% w/v in saline) and placed on the dowel. At this dose, these animals were unable to balance on the dowel. Five minutes after the mouse fell off of

the dowel, it was tested (and every 5 min thereafter) until it was able to remain on the dowel for >1 min. with recovery time recorded as time from loss of balance to time of balance recovery.

Loss-of righting reflex assay—Ethanol (10% w/v in saline) was administered IP in different volumes to obtain three test doses (3.2, 3.6 or 4.0 g/kg). After injection, mice were placed on their backs and tested for loss of righting reflex. The mouse was judged to have lost the righting reflex at the time when it could not right itself three times within 30 sec. When the animal was able to right itself three times within 30 sec. it was deemed to have recovered. The duration of the loss of the righting reflex was calculated as the difference between when the reflex was lost and when it was recovered. Mice were tested at all three doses (3.2, 3.6 and 4.0 g/kg) of ethanol using a random order balanced design, with a week interval between each test session.

Ethanol metabolism and clearance—A separate group of ethanol-naïve mice were injected with 4g/kg of ethanol and tail blood samples (10 µl) obtained at 30, 60 and 120 minutes to measure blood alcohol levels. Blood ethanol content was assessed from serum using the Analox AM1 Analyzer (Analox Instruments, North Yorkshire, UK).

Two-bottle choice assay—Oral alcohol self-administration and preference were examined using a two-bottle choice protocol. Water intake (ml) and body weight (g) were recorded on alternate days for 4 days to test potential differences in fluid consumption. During this period, water was the only fluid available, but from 2 separate bottles. The animals were then given a choice between ethanol (3% w/v) and water. Two-bottle drinking sessions were conducted 23 h per day, 7 days per week. During the course of the exposure period, ethanol concentration was increased from 3% to 20% (3, 6, 10, 14, 20%) with four days access at each concentration. On alternate days, the mice were weighed and placed in individual holding chambers while the fluids were replaced in the home cage. The position (left or right) of each solution was alternated every two days to control for side preferences. One week after the ethanol self-administration procedure, the same mice were tested for saccharin (sweet) and quinine (bitter) intake and preference also using a two-bottle choice protocol. Each tastant was provided in water at 2 concentrations (saccharin: 0.03% and 0.06% w/v; quinine: 0.015mM and 0.03mM) and tested at each concentration for 2 days.

Statistical Analysis

C. elegans data were analyzed using Graphpad Prism software with the following statistical tests performed: dispersal data: 2-way ANOVA followed by Bonferroni posttests, where appropriate; locomotion and internal ethanol data: paired two-tailed t-test.

Statistical analysis of mouse behavioral data was performed using SigmaStat 3.1, with appropriate post-hoc comparisons performed as indicated by SigmaStat. Statistical tests were performed as follows: loss of righting reflex assay: 2-way ANOVA for genotype x dose, followed by Newman-Keuls posthoc comparison; ethanol metabolism and clearance: 2-way repeated measures ANOVA for genotype x time; stationary dowel assay: 1-way ANOVA for genotype; 2-bottle choice assay: 2-way repeated measures ANOVA for genotype x ethanol concentration, followed by Tukey posthoc comparison. Ethanol preference data were further analyzed within genotype using 1-way repeated measures ANOVA for ethanol concentration.

RESULTS

We tested existing *C. elegans* mutants with synaptic defects for altered responses to ethanol using a locomotor dispersal assay, which quantifies the ability of worms to move toward a bacterial food source on an agar plate (Crowder *et al.*, 1996). We observed that *rab-3* mutants exhibit significant resistance to ethanol in the dispersal assay when treated with 400 mM exogenous ethanol while dispersing normally in the absence of ethanol (Fig. 1A–C). This was observed with two different null alleles, *js49* and *y250*.

Although the dispersal assay provides a means of rapidly assessing the ethanol sensitivity of different strains, changes in the dispersal assay could arise through effects of ethanol on behaviors other than locomotion. For instance, movement of animals to bacteria is in part mediated by chemosensory neurons that are sensitive to odorants in the environment. An effect of ethanol on the activity of these neurons could influence the dispersal of animals in these assays. To more directly analyze the effects of ethanol specifically on the locomotor behavior of *rab-3* mutants, we also quantified the speed of movement of animals using image analysis software. The software allows simultaneous tracking of multiple animals on the same test plate. The locomotion rates of wild-type and *rab-3* animals were compared on the same plate (in the absence of food) using metal rings to isolate the groups of animals. In this assay, *rab-3(js49)* and *rab-3(y250)* showed significant resistance to ethanol (Fig. 1D, $P < 0.05$). We also analyzed *rab-3(js49)* and wild-type control animals treated with 400mM ethanol for internal ethanol concentration and found no strain effect, indicating that ethanol absorption/metabolism is normal in *rab-3* mutants. (Fig. 2).

RAB-3 regulates synaptic vesicle trafficking in its active, GTP-bound form (Geppert *et al.*, 1994). Although the process by which this occurs is poorly understood, several RAB-3-interacting proteins have been identified. One of these, AEX-3, is a RAB-3 guanine nucleotide exchange factor (Iwasaki *et al.*, 1997). Loss of AEX-3 is expected to shift the equilibrium from active, GTP-bound RAB-3 to inactive, GDP-bound RAB-3, and should therefore mimic the effect of loss of RAB-3 function. We tested three *aex-3* loss-of-function alleles (*y255*, *sa5* and *n2166*) for sensitivity to the sedative effects of ethanol on locomotion. As predicted, disruption of *aex-3* in *C. elegans* phenocopied the resistance to ethanol observed in *rab-3* mutants (Fig. 1D, $P < 0.05$).

We more fully characterized ethanol's mechanism of action in the *rab-3* and *aex-3* pathway. We tested the specificity of the ethanol resistance generated by loss of function in *rab-3* and *aex-3* by examining loss of function in another gene (*cab-1*) that functions in neurons with *aex-3* but independently of *rab-3*. *cab-1* encodes a protein that binds AEX-3 and has been shown to partially mediate aspects of the phenotype associated with mutations in *aex-3* (Iwasaki and Toyonaga, 2000). Comparison of the ethanol sensitivity of *cab-1(tg46)* mutant animals with wild-type animals revealed no difference in the effect of ethanol on speed of locomotion ($t_4 = 1.14$, $P = 0.32$) (Fig. 1D). The lack of ethanol resistance in the *cab-1* mutant animals suggests that the resistance associated with *aex-3* is due to its functional interaction with *rab-3*.

To determine if the role of *rab-3* in the behavioral response to ethanol is conserved in mammals, we assayed mice carrying a previously described *Rab3A* null allele (Geppert *et al.*, 1994) for altered sensitivity to the acute ataxia-inducing effects of a low, 1.5mg/ml dose ethanol. We observed a significantly shortened recovery time in both *Rab3A*^{−/−} and *Rab3A*^{+/-} mice relative to *Rab3A*^{+/+} controls ($n = 12$ – 14 ; $P < 0.01$) (Fig. 3A). Although not significant, we observed a trend among *Rab3A*^{+/-} and *Rab3A*^{−/−} mice toward increased latency to initial loss of balance in this assay, also indicative of ethanol resistance (data not shown). Mice were also tested for sensitivity to the acute sedative/hypnotic effects of

ethanol in the loss-of-righting reflex assay. We observed a significant reduction in recovery time for both *Rab3A*^{-/-} and *Rab3A*^{+/-} mice relative to *Rab3A*^{+/+} controls at the 4g/kg dose ($n = 9-14$; *Rab3A*^{+/+} vs. *Rab3A*^{+/-}, $P < 0.01$; *Rab3A*^{+/+} vs. *Rab3A*^{-/-}, $P < 0.001$) (Fig. 3B). A drug naïve cohort of subjects was used to assess ethanol metabolism/clearance of a 4g/kg IP injection of ethanol. We failed to detect a significant main effect for genotype ($F[2, 15] = 0.41$, $P = 0.67$), indicating that *Rab3A* mutant mice metabolize ethanol normally (Fig. 3C). Thus, loss of either one or two copies of *Rab3A* results in resistance to the acute ataxic and sedative effects of ethanol.

Studies in humans and several mouse genetic models have established a positive correlation between resistance to the acute effects of ethanol and the propensity for alcohol consumption/abuse (Schuckit, 1994). We therefore assessed voluntary ethanol consumption in the *Rab3A* mutant mice using the two-bottle choice assay. Interestingly, we observed overdominant effects in the heterozygotes: *Rab3A*^{+/-} mice showed increased consumption of 14% and 20% ethanol solutions ($n = 10-13$, $P < 0.001$ for 14% ethanol, $P < 0.005$ for 20% ethanol). Across all ethanol concentrations, a significant genotype x solution interaction was observed ($F[8, 272] = 5.16$, $P < 0.001$; posthoc comparisons of genotype within 14%: $P < 0.001$ and within 20%: $P < 0.005$) (Fig. 4A). Although we failed to detect a significant strain effect for ethanol preference, we observed a significant genotype x concentration interaction ($F[8, 272] = 2.26$, $P < 0.05$, Fig. 4B). Further analysis revealed that ethanol preference in *Rab3A*^{+/-} mice was not dependent upon ethanol concentration ($n = 10$, $P = 0.20$) in contrast to *Rab3A*^{+/+} and *Rab3A*^{-/-} mice, which showed a significant concentration-dependent effect on preference ($n = 11-13$, $P < 0.001$ and $P < 0.005$, respectively). We observed no strain effect for consumption or preference for water, saccharin solution, or quinine solution, indicating that the increased ethanol consumption and altered preference were not due to altered taste preference or liquid consumption (Fig. 4C, D).

DISCUSSION

The data presented here show that loss-of function mutations in *rab-3* and the *rab-3* exchange factor *aex-3* confer resistance to the locomotor effects of ethanol in *C. elegans*. Furthermore, we demonstrate that disruption of the *cab-1* gene, which encodes a presynaptic protein that associates with AEX-3 (Iwasaki and Toyonaga, 2000), fails to phenocopy the ethanol resistance of *rab-3* and *aex-3* mutants, suggesting that the behavioral effect is specific to RAB-3 function. The lack of *cab-1*-associated ethanol resistance also minimizes the possibility that the resistance we observe with *rab-3* and *aex-3* mutants is due to a general effect on synaptic transmission; *cab-1* mutants, like *rab-3* and *aex-3* mutants, display increased resistance to the acetylcholinesterase inhibitor aldicarb, a phenotype that is indicative of decreased levels of synaptic transmission (Iwasaki *et al.*, 1997; Iwasaki and Toyonaga, 2000; Nonet *et al.*, 1997). If a decrease in levels of synaptic transmission were a general mechanism for ethanol resistance, then we would expect all three mutants to show resistance. That is not the case; in fact, we have shown previously that mutations in the *slo-1* gene, which increase synaptic transmission, also result in ethanol resistance (Davies *et al.*, 2003). This suggests that the ethanol phenotype does not simply reflect a nonspecific alteration in synaptic transmission.

A potential mechanism for nonspecific resistance to the behavioral effects of ethanol is a change in the penetration of ethanol into the animal or altered metabolism of ethanol. Such a mechanism does not appear to contribute to the resistance of *rab-3* mutants because we found that tissue concentrations of ethanol in *rab-3* mutant animals are equivalent to those observed in wild-type animals when the strains are exposed to the same exogenous dose of ethanol under the same conditions in which they were tested for locomotion. It is worth

noting that internal tissue concentrations of ethanol are substantially lower than exogenous ethanol concentrations (also see Davies *et al.*, 2003, 2004). The lower tissue concentrations resulting from these high exogenous doses reflects the lack of permeability of *C. elegans* and has also been observed in studies of other neuroactive compounds (Rand and Johnson, 1995). These tissue concentrations of ethanol are the same as those resulting in intoxication in vertebrate systems, including humans (Diamond and McIntire, 2002).

Consistent with our behavioral data in *C. elegans*, we show that *Rab3A*^{+/-} and *Rab3A*^{-/-} mice are acutely resistant to the ataxic and sedative effects of ethanol. These data do not address whether the resistance to ethanol in both species is the result of altered acute sensitivity or abnormal development of tolerance. It may be noted that prior to the sedation and ethanol consumption assays, our subjects were not ethanol naïve (ie, mice had previously been exposed to low doses of ethanol for the ataxia assay), so it is possible that the altered sedation and/or consumption phenotypes we observe have been affected by RAB3A-dependent differences in tolerance. It will be important for future studies to investigate what role, if any, RAB3A has in the development of ethanol tolerance. Interestingly, we failed to observe a strain effect for the loss-of righting reflex test at a low ethanol dose (3.2g/kg), possibly reflecting a floor effect of recovery time on sedation at this dose in these animals (i.e., all genotypes showed a minimal recovery time of approximately 20 minutes at this dose), whereas a trend toward reduced recovery time was observed in mutants at the 3.6g/kg dose. One might predict a more robust shift in the dose response curve in *Rab3A* mutant mice on a more sensitive genetic background.

Previous studies in *C. elegans* have shown that synaptic transmission is impaired in *rab-3* (*js49* and *y250*) mutants, an observation attributed to alterations in either RAB-3 regulated recruitment of synaptic vesicles to the active zone and/or their sequestration near release sites (Nonet *et al.*, 1997). Electrophysiological studies of *Rab3A* null mice have established a regulatory role for RAB3A in Ca²⁺-dependent synaptic vesicle release (Geppert *et al.*, 1994, 1997), although its precise function(s) remains unclear. In mammals, four related RAB3 proteins have been shown to be partially functionally redundant, complicating the analysis of RAB3A function (Schluter *et al.*, 2004). Interestingly, RAB3 proteins in the mouse, in contrast to in *C. elegans*, do not appear to be required for synaptic vesicle trafficking to the active zone *per se*, but rather may affect neurotransmitter release by increasing the release probability of a subset of primed vesicles (Nonet *et al.*, 1997; Schluter *et al.*, 2006). Additional evidence suggests that mammalian RAB3A may also be involved in regulating trafficking of recycled synaptic vesicles (Star *et al.*, 2005).

Although small GTP binding (RAB) proteins have not previously been implicated in behavioral sensitivity to ethanol, there is evidence that RAB proteins regulate vesicular trafficking in hepatocytes in response to ethanol treatment (Larkin *et al.*, 1996). Moreover, ethanol-induced disruption of the secretory machinery has been reported to alter RAB protein levels in astrocytes and the pituitary (Ren *et al.*, 2005; Tomas *et al.*, 2005). Ethanol may similarly affect vesicular trafficking or exocytosis in neurons through a RAB-3/RAB3A dependent mechanism, although we have no direct evidence to support this.

Ethanol has been shown to indirectly inhibit the release of neurotransmitter through modulation of neural activity. Ethanol activates presynaptic BK potassium channels in *C. elegans* and vertebrates (Cowmeadow *et al.*, 2005, 2006; Davies *et al.*, 2003; Gruss *et al.*, 2001), inhibiting neurotransmitter release. Similarly, inhibition of presynaptic N/P/Q-voltage gated calcium channels by ethanol reduces vesicular release (Maldve *et al.*, 2004). One might expect that disruption of RAB-3/RAB3A function in the mutants would impair neurotransmitter release further, potentially resulting in an enhanced response to ethanol or hypersensitivity. However, the unexpected resistance of *rab-3/Rab3A* mutants to ethanol

suggests that instead ethanol may exert additional inhibitory effects on RAB3-dependent neurotransmitter release, downstream of BK and Ca²⁺ channel function.

We observed that *Rab3A*^{+/-} mice voluntarily consume significantly more ethanol than wild-type or *Rab3A*^{-/-} mice and that ethanol preference is not dependent on ethanol concentration in *Rab3A*^{+/-} mice. Based on human and rodent studies indicating that initial sensitivity predicts subsequent ethanol consumption (Boyce-Rustay *et al.*, 2006; Crabbe *et al.*, 1996; Fee *et al.*, 2004; Naassila *et al.*, 2002, 2004; Newton and Messing, 2007; Offenhauser *et al.*, 2006; Palmer *et al.*, 2004; Schuckit, 1994; Thiele *et al.*, 2000), we would have expected *Rab3A*^{-/-} mice to consume more ethanol than wild-type animals. Instead, it appears that ethanol sensitivity and consumption are dissociated in *Rab3A*^{-/-} mice. Why we observe increased ethanol consumption in *Rab3A*^{+/-} mice but not *Rab3A*^{-/-} mice is unclear. One possible explanation is that in the absence of RAB3A protein, compensatory changes may occur in other RAB proteins that do not occur in the *Rab3A* heterozygotes. Some degree of functional redundancy among RAB3 genes has been observed with respect to embryonic viability (Schluter *et al.*, 2004). Behaviorally, such an effect would appear to be specific to ethanol consumption, as we observe resistance to some of the acute effects of ethanol in *Rab3A*^{-/-} mice. It may be noted that a similar overdominance effect on ethanol-related behaviors has been reported for other genes: dopamine transporter (DAT) haploinsufficiency in female mice results in increased voluntary ethanol consumption relative to wild-type and homozygous null mice (Savelieva *et al.*, 2002). *Gat1* (GABA transporter subtype 1) haploinsufficiency has also been reported to result in increased ethanol consumption in mice, whereas *Gat1*^{-/-} mice consume normal amounts of ethanol (Cai *et al.*, 2006). It is possible that the ethanol consumption phenotype of *Rab3A*^{+/-} mutants reflects altered dopaminergic and/or GABAergic neurotransmission.

Behaviorally, *Rab3A* disruption in the mouse has been shown to affect circadian activity and sleep homeostasis (Kapfhamer *et al.*, 2002) with conflicting reports of altered performance in certain learning and memory tasks (D'Adamo *et al.*, 2004; Hensbroek *et al.*, 2003; Yang *et al.*, 2007). Our data extend the behavioral role of RAB3A to include the regulation of ethanol-related behaviors and suggest a possible novel mechanism underlying the behavioral response to ethanol.

Acknowledgments

The authors thank M. Ferwerda, M. Wallace and J. Connolly for technical assistance and members of the McIntire, Heberlein and Messing labs for helpful discussions. This work was supported by the State of California for medical research on alcohol and substance abuse through UCSF, and by the NIAAA, NIH (J.C.B., A.G.D., S.L.M., U.H.), ABMRF (A.G.D.) and by the Jeffress Memorial Trust (J.C.B.).

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Figure 1.

rab-3 mutants are resistant to locomotor depressant effects of ethanol. **A–C**, Dispersal indices (D.I.s) of wild type (N2) control, *rab-3(js49)* and *rab-3(y250)* mutants on 0, 200, and 400mM ethanol. **D**, *rab-3(js49)* and *y250* and *aex-3(y255)*, *sa5* and *n2166* mutants move significantly faster than wild-type (N2) controls when treated with 400mM ethanol. In contrast, *cab-1(tg46)* mutants have wild-type ethanol sensitivity. Error bars are mean $\pm$ SEM. Asterisks in **C** indicate levels of statistical significance (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Figure 2.

rab-3 mutations do not alter ethanol pharmacokinetics in *C. elegans*. The internal ethanol concentration of worms was analyzed at 20 minutes of exposure to an exogenous ethanol dose of either 0mM or 400mM. Internal ethanol concentrations are substantially lower than exogenous ethanol concentrations. Internal ethanol concentrations of N2 and *rab-3(js49)* are not significantly different from each other at either 0mM or 400mM as tested by paired two-tailed t-test ($t_3 = 1.71$, $P = 0.19$).

Figure 3.

Disruption of *Rab3A* in the mouse results in altered ethanol-dependent behaviors. **A**, *Rab3A*^{-/-} and *Rab3A*^{+/-} mice are resistant to the ataxic effects of 1.5g/kg ethanol in the stationary dowel test. **B**, *Rab3A*^{-/-} and *Rab3A*^{+/-} mice are resistant to the sedative effects of 4g/kg ethanol in the LORR assay. **C**, Normal ethanol metabolism of 4g/kg IP dose is observed in *Rab3A* mutant mice; BEC = “blood alcohol content”. Error bars are mean $\pm$ SEM.

Asterisks in **A** and **B** indicate levels of statistical significance (*, $P < 0.01$; **, $P < 0.001$).

Figure 4.

Altered ethanol consumption and preference in *Rab3A*^{+/-} mice. **A**, *Rab3A* haploinsufficiency results in increased voluntary ethanol intake. **B**, Ethanol preference of *Rab3A*^{+/-} mice is not dependent on ethanol concentration, in contrast to *Rab3A*^{+/+} and *Rab3A*^{-/-} mice. **C**, Normal water consumption is seen in *Rab3A* mutant mice. **D**, Normal saccharin and quinine preference in *Rab3A* mutant mice. Error bars are mean $\pm$ SEM. Asterisks in **A** indicate levels of statistical significance (**, $P < 0.005$).