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Supplemental Data

Distinct Behavioral Responses to Ethanol Are Regulated by Alternate RhoGAP18B Isoforms

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Supplementary Experimental Procedures

Ethanol Absorption

To test whether the resistance to sedation observed with *whir* mutant flies was caused by a change in ethanol absorption, we measured the internal alcohol concentration of flies exposed for 8 minutes to a high ethanol concentration (E/A = 120/30). We chose this short exposure time because sedated flies absorb ethanol more readily than flies that are active (A. Rodan, A. Corl, and U.H., unpublished observation). After an 8-min exposure, neither $whir^1$ nor wild-type flies were sedated. Internal ethanol concentrations were 38.2 ± 1.0 mM for $whir^1$ and 41.5 ± 2.7 mM for wild-type flies, a difference that was not significant ($p>0.32$, $t=1.1$, t-test, $n=4$). The absorption was determined as described in Moore et al. (Moore et al., 1998) using the Alcohol Reagent 20 from Sigma Diagnostics (St. Louis, MO).

Behavioral Tests

Circadian rhythm period lengths in constant darkness were determined as described in Cyran et al. (Cyran et al., 2003), and were no different between wild-type (tau = 23.6 ± 0.2 hours, n=11) and $whir^1$ flies (tau = 23.8 ± 0.1 hours, n=7).

Quantitative RT-PCR

The amplification primers for quantitative RT-PCR were primer pairs Dm01844423_m1 (Applied Biosystems, Foster City, CA) for RA, 5’GAGTGTGCGTGGCTTTCTCA and 5’GCCACAGCCACAAGCTGAA for RC, and 5’CGGAGTGCGCCATAAGTTGT and 5’TCGGCATCATCATCTTGGAA for *RC&RD*. Probe sequences were 5’ACGGCTTGAGGATTGTGCCAGTGAC for RA, 5’CGCCTATGACAAATC for RC, and 5’CACATCGACGCTGACCA for RC&RD. The primers used for the *Tubulin 84B* northern probe spanned the first intron: 5’ACAGCTTGCCGTCTCTAGCTCCG (5’) and 5’GCAGGCGTTTCCAATCTGGACACC (3’). For *RhoGAP18B*, the probe was amplified from reverse-transcribed head RNA with 5’GCAACCTGCTCGATCTGCCCG and 5’CTGGCCTCGACGTGAACGCG and recognizes all transcripts near the 5’ end of the GAP domain.

In Situ Hybridization

To determine if the GAL4 expression pattern recapitulated the endogenous *RhoGAP18B* expression pattern, we carried out in situ hybridization experiments with *RC*- and *RA*-specific probes. In the adult head, the *RC* transcript appeared to be expressed ubiquitously (data not shown), while *RA* expression was too low to be detected reliably (which is consistent with our Northern blots, Fig. 2B). *RA* is, however, expressed in the adult fly head as it was readily detectable by RT-PCR (Fig. 2C and data not shown). Thus, the GAL4 enhancer-traps capture only a subset of the endogenous RC expression pattern, a pattern that is, however, of functional relevance. Outside of the nervous system, *whir*^{1} drives expression in the salivary glands and a few cells along the gut; similar expression was seen by in situ hybridization using *RC* and *RA* specific probes (data not shown).

Supplemental References

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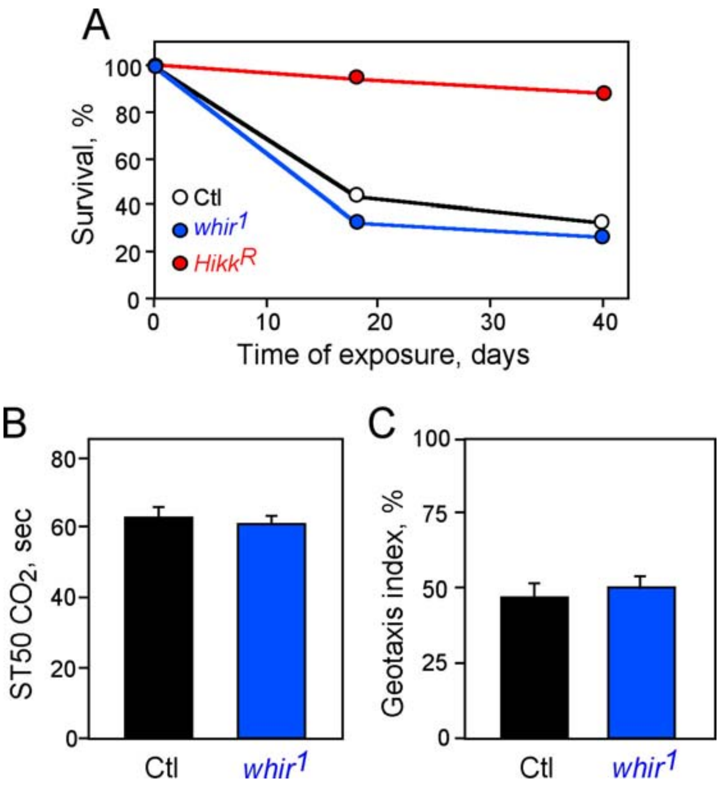

Figure S1. Behavioral Responses Not Affected by the *white rabbit* Mutation

(A) *whir* flies show normal sensitivity to the toxic effects of nicotine. The fraction of flies surviving on nicotine-containing food (1 mg/ml) as a function of time is shown. Wild-type and *whir*^{1} flies were equally sensitive to chronic nicotine exposure in the food, while a strain that overexpresses Cyp6g1, and is resistant to various insecticides (Hikkone-R) (Daborn et al., 2001), showed resistance (p=0.1 of wild type *vs whir*^{1} at 18 hours, and p<0.01 of Hikk R vs wild type or vs *whir*^{1} at 18 hours, chi-square test, n=50-100 flies).

(B) *whir* flies show normal sensitivity to CO_2 exposure. Time required for 50% of the flies to lose their righting reflex is shown. Exposure to CO_2 was done using a very low flow rate, barely noticeable to the cheek. This constant setting was successively used on multiple vials of 6 flies, which were in random order, unknown to the experimenter. (p=0.6, t=0.5, t-test, n=4 experiments).

(C) Negative geotaxis, as measured in the counter-current apparatus (Benzer, 1967), was normal in *whir* mutant flies. Briefly, 25 flies were given 15 seconds to climb up a tube of about 20 cm in length. This was repeated 6 times, and flies climbing into the upper half each time received a score of 1, flies never reaching the upper half scored 0. The score of one experiment consisted of the average score of 25 flies. Wild-type and *whir*^{1} mutant flies climb equally well (p=0.6, t=0.5, t-test, n=5 experiments). All data are means±SEM.

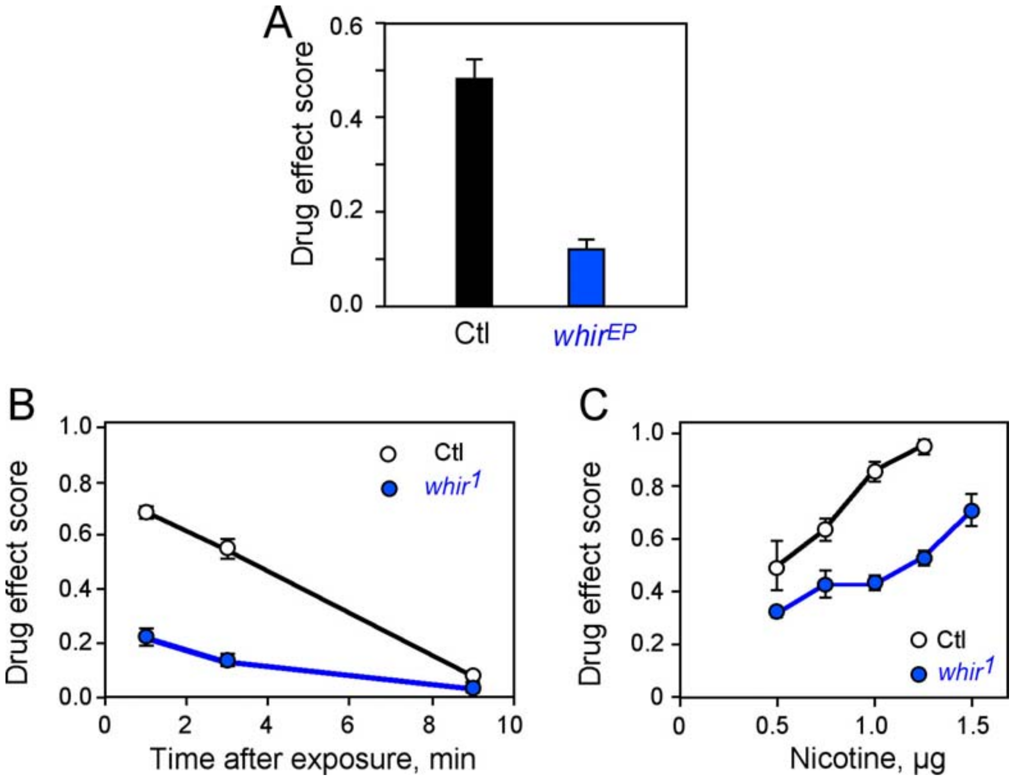

Figure S2. Cocaine and Nicotine Resistance of *white rabbit* Flies

(A) Wild-type and *white rabbit* (allele *EP1439*) mutant flies were exposed to 150 μg cocaine and their negative geotaxis was measured in the crackometer (Bainton et al., 2000). A drug effect score of 0 indicates that flies are unaffected by the drug, while a score of 1 indicates that all flies are unable to climb the walls of the cylinder. *whir* mutant flies are resistant to the incapacitating effects of cocaine ($p<0.001$, $t=7.5$, t-test, $n=13$). The *EP(X)1439* P-element inserts at the same position in the genome as *whir*^{2}.

(B) Time course of the ability to perform negative geotaxis after exposure to 0.8 μg nicotine. *whir*^{1} mutant flies show resistance to the effects of nicotine ($p<0.001$, $t=11.3$, t-test, $n=8$ experiments).

(C) Dose-response curve of the drug effect score quantified one minute after nicotine exposure. *whir*^{1} flies are less affected than wild-type flies at all nicotine doses tested ($p<0.05$, t-test, $n=4$ experiments). All data are means±SEM.

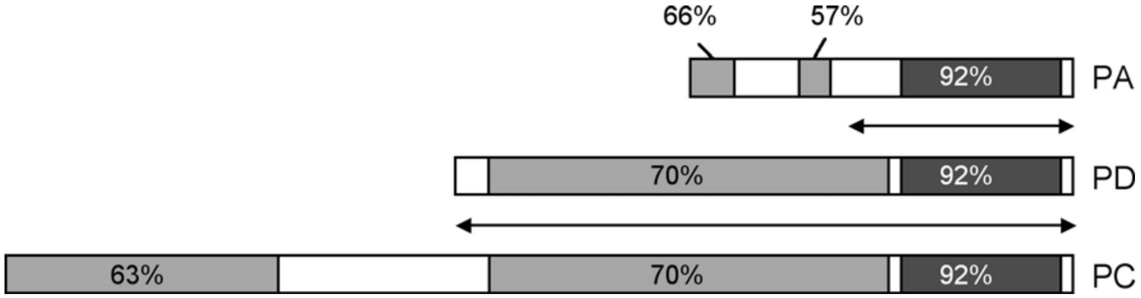

Figure S3. Structure and Conservation of RhoGAP18B Proteins

Predicted structures for the three proteins, PA, PD, and PC, encoded by the RhoGAP18B locus. Domains shared among the different proteins are indicated by the arrows between the protein schematics. The percent identity between *D. melanogaster* and *D. pseudoobscura* are indicated for stretches of >50% identity.

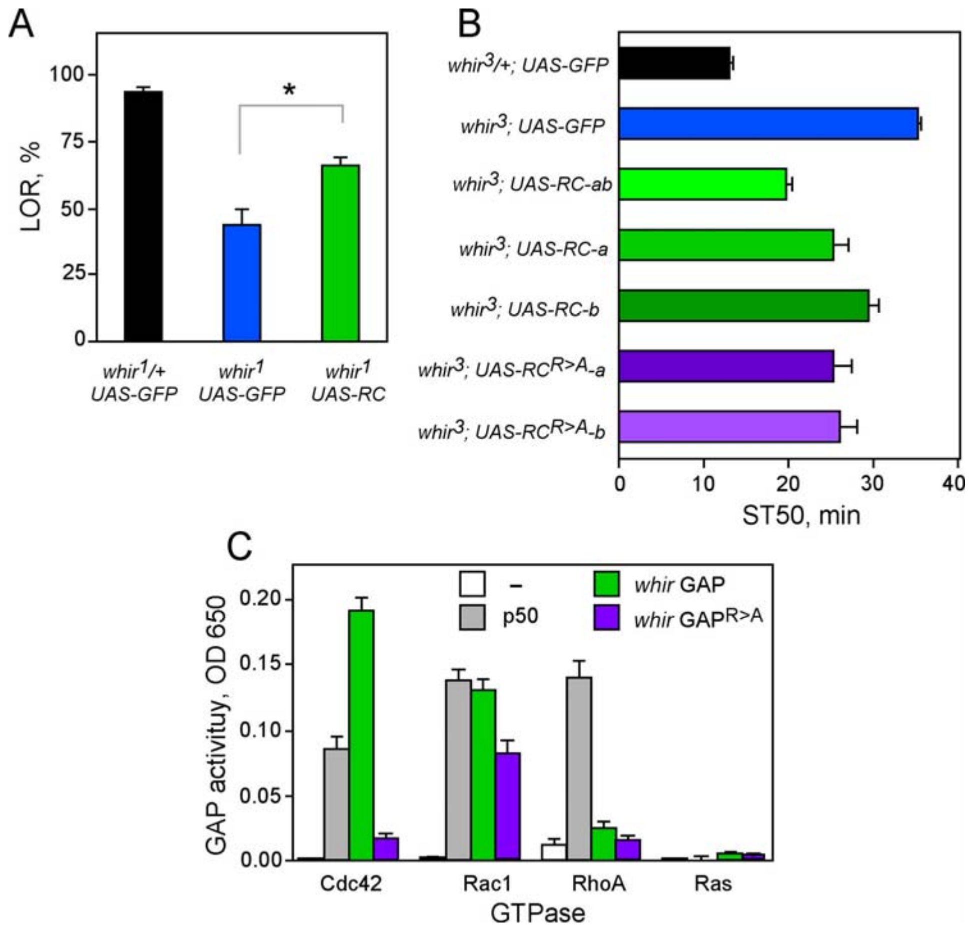

Figure S4. Rescue of Ethanol-Induced Sedation

(A) Phenotypic rescue of the *whir*^{1} allele. The fraction of flies that show loss-of-righting (LOR) after 31 minutes of exposure to ethanol (E/A = 120/30) is shown. *whir*^{1} flies expressing the RC-cDNA (*whir*^{1}/+;*UAS-RC*) show a significant rescue of sedation resistance towards wild type (*whir*^{1}/+;*UAS-GFP*)($p<0.01$, t=3.8, n=6 experiments). This phenotypic rescue was weaker than what was achieved with the *whir*^{3} driver (Fig. 5), presumably due to the fact that GAL4 expression driven by the *whir*^{1} driver is lower than that observed with the *whir*^{3} driver (data not shown).

(B) Phenotypic rescue is dose-dependent and can be achieved with a ‘mutant’ *RC* transgene. Time to 50% loss-of-righting (ST50) after exposure to ethanol (E/A =110/40) is shown. The ST50 for wild-type flies (*whir*^{3}/+;*UAS-GFP*) was 13.3 min, while that for mutant *whir*^{3} flies (*whir*^{3}; *UAS-GFP*) was 36 min ($p<0.001$, n=5, LSD test). Mutant flies carrying 2 copies of the *RC*-cDNA (*whir*^{3}; *UAS-RC*) showed a substantial normalization of ST50 (to 20 min) when compared to wild type ($p<0.001$, n=5-6, LSD test). The phenotypic rescue, although still statistically significant, was less pronounced in *whir*^{3} flies carrying only one of the *UAS-RC* transgenes (*UAS-RC-a* or *UAS-RC-b*) ($p<0.001$, n=6 for *whir*^{3};*UAS-RC-a vs whir*^{3}*;UAS-GFP*. $p<0.003$, n=5-6 for *whir*^{3};*UAS-RC-b vs whir*^{3};*UAS-GFP*). Introducing a point mutation in the catalytic arginine of the GTPase activating domain encoded by the *RC* transcript had no effect on rescue efficacy; expression of two mutant transgenes (*UAS-RC*$^{R>A}$-a and *UAS-RC*$^{R>A}$-b) in the *whir*^{3} pattern significantly ameliorated the ethanol-sedation defect of *whir*^{3} flies ($p<0.001$, $n=5$ for both *vs whir*^{3}*;UAS-GFP*). All *RC*-cDNA constructs used rescued the semi-lethality associated with the *whir*^{3} strain to similar extents (data not shown).

(C) GTPase stimulating activity of a RhoGAP18B GAP domain carrying a mutation in the catalytic arginine to alanine (R>A). This mutation drastically reduced the stimulation of Cdc42 GTPase, but affected activation of Rac1 only marginally. These data are consistent with the fact that transgenes expressing the mutant GTPase are still able to rescue the ethanol sedation defect of *whir* flies (panel B). The data shown, with the exception of GAP$^{\mathrm{R>A}}$, are the same as displayed in Fig. 3A and are shown for comparison. All data are means±SEM.

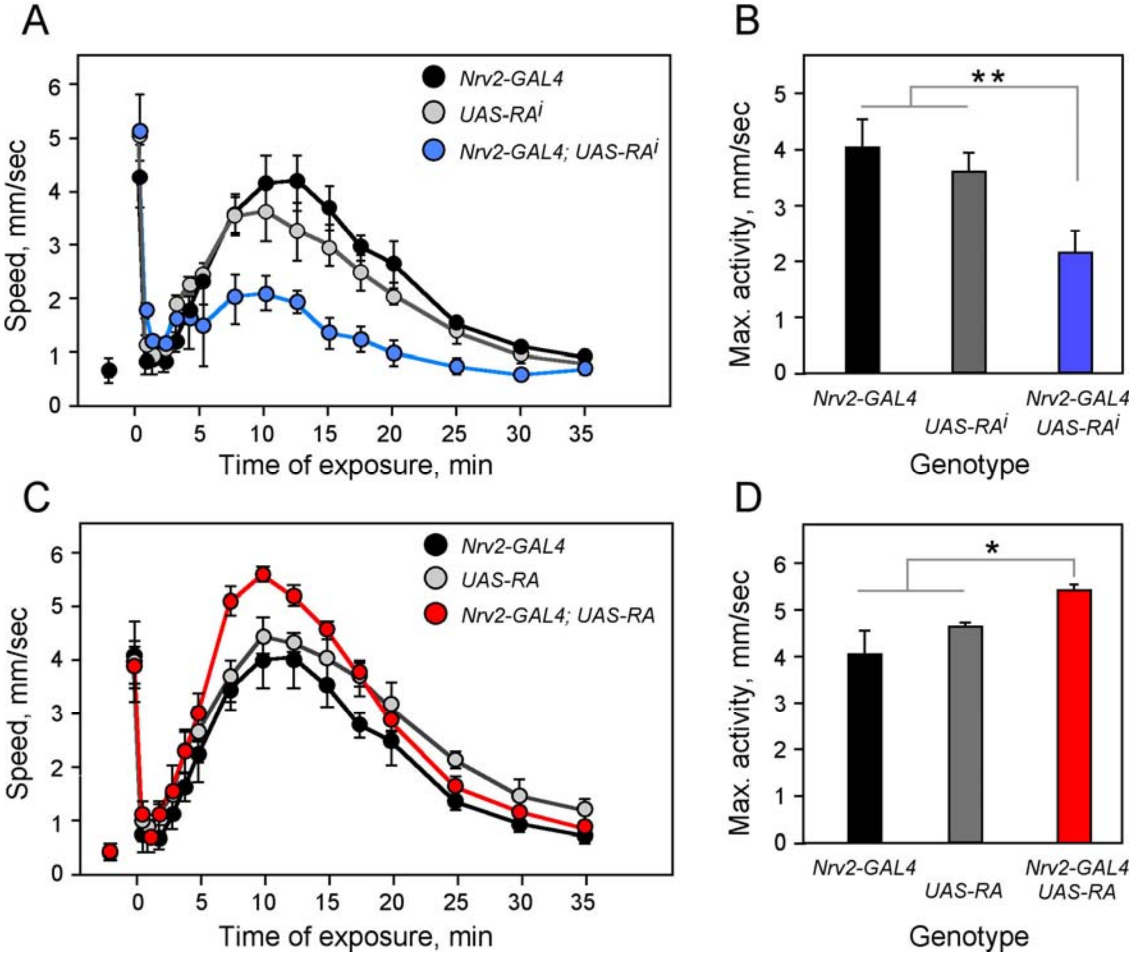

Figure S5. Pan-Neural Manipulations of *RhoGAP18B-RA* Transcript with the *Nrv-Gal4* Driver Affect Hyperactivity

(A-D) Locomotion tracking (E/A = 100/50) of flies with increased or reduced expression of *RA* transcript in the nervous system. *RA* down-regulation (*Nrv2-GAL4;UAS-RNA*$^{\mathrm{i}}$) using the *Nrv-GAL4* driver (A, B) (Sun et al., 1999) led to significantly decreased hyperactivity (*p<0.05, t=2.9 and 3.0 *vs* both controls, t-test, n=4-5 experiments). Hyperactivity was significantly increased upon *RA* over-expression (*Nrv2-GAL4;UAS-RA*) (C, D) (*p<0.05, t=2.6 and 3.1, t-test, n=4-5 experiments). The *Nrv2-GAL4* control flies were the same in (A, B) and (C, D). A second, independent *UAS-RA*^{i} showed essentially the same results (data not shown). Note that expression of *RA* transgenes has no effect on either baseline locomotion prior to ethanol exposure, or on the ethanol-induced startle response in the first minute of exposure, and the effect is therefore specific to the ethanol-induced hyperactivity phase. Data are means±SEM.

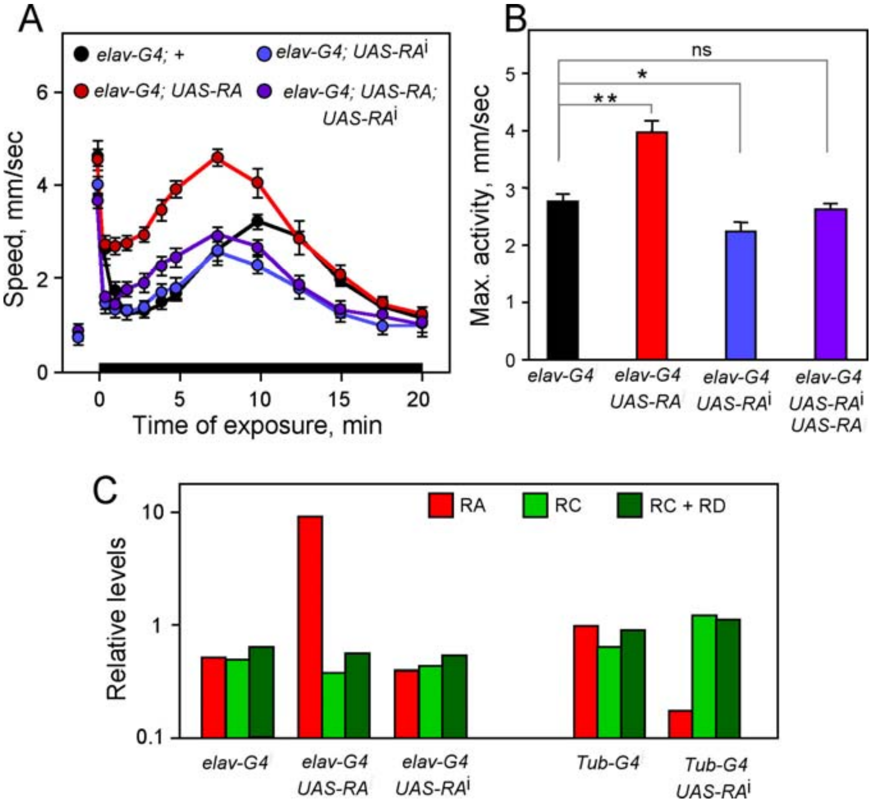

Figure S6. Pan-Neural Manipulations of *RhoGAP18B-RA* Transcript with the *elav-GAL4* Driver Affect Hyperactivity

(A, B) Manipulations of *RA* transcript levels pan-neurally affect ethanol-induced hyperactivity. Locomotion tracking (E/A = 100/50) of flies with increased or reduced expression of *RA* transcript in the nervous system using the *elav-GAL4* driver (Robinow and White, 1991). *RA* down-regulation (*elav-GAL4;UAS-RA*^{i}) led to significantly decreased hyperactivity (*p<0.02 vs control, LSD test, n=7-9). Hyperactivity was significantly increased upon RA over-expression (*elav-GAL4;UAS-RA*) (**p<0.001). The hyperactivity of flies expressing both the *RA* cDNA and the RNAi transgene simultaneously (*elav-GAL4;UAS-RA;UAS-RA*^{i}) did not differ significantly from wild-type controls (*elav-Gal4;+*, p=0.37), indicating molecular competition between the two transgenes. Note that expression of RA transgenes had no effect on either baseline locomotion prior to ethanol exposure, or on the ethanol-induced startle response in the first minute of exposure, the effect is therefore specific to the ethanol-induced hyperactivity phase. The data are means±SEM.

(C) Quantitative RT-PCR analysis of *RhoGAP18B* transcript levels. *RC* transcript levels were unaffected in the *RA* transgenic lines. Pan-neuronal RA expression (*elav-Gal4;UAS-RA*) led to an approximately tenfold increase in *RA* transcript, while expression of the RNAi transgene (*elav-Gal4;UAS-RA*^{i}) did not measurably lower *RA* levels. However, driving the *UAS-RA*^{i} transgene with a strong ubiquitous GAL4 driver, *Tub-GAL4*, resulted in a five- to six-fold reduction of *RA*, but not *RC* transcript levels in larvae (transcript levels were quantified in larvae as the experimental flies die late in larval life). Thus, the *UAS-RA*^{i} transgene is active, but its effect upon expression in the nervous system is too weak to be detected molecularly.