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Genetic Dissociation of Ethanol Sensitivity and Memory Formation in *Drosophila melanogaster*

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ABSTRACT

The *ad hoc* genetic correlation between ethanol sensitivity and learning mechanisms in Drosophila could overemphasize a common process supporting both behaviors. To challenge directly the hypothesis that these mechanisms are singular, we examined the learning phenotypes of 10 new strains. Five of these have increased ethanol sensitivity, and the other 5 do not. We tested place and olfactory memory in each of these lines and found two new learning mutations. In one case, altering the *tribbles* gene, flies have a significantly reduced place memory, elevated olfactory memory, and normal ethanol response. In the second case, mutation of a gene we name ***e**thanol sensitive* with ***l**ow **m**emory* (*elm*), place memory was not altered, olfactory memory was sharply reduced, and sensitivity to ethanol was increased. In sum, however, we found no overall correlation between ethanol sensitivity and place memory in the 10 lines tested. Furthermore, there was a weak but nonsignificant correlation between ethanol sensitivity and olfactory learning. Thus, mutations that alter learning and sensitivity to ethanol can occur independently of each other and this implies that the set of genes important for both ethanol sensitivity and learning is likely a subset of the genes important for either process.

THERE are at least some common mechanisms in learning and behavioral responses to ethanol, although the number of genes with deficits in both behaviors is small and testing in some cases has been *ad hoc*. Thus, the idea of a mechanistic singularity in these behaviors might be overstated. To address potential complexities in the mechanisms that support the ethanol response and learning, we tested for learning phenotypes in new Drosophila strains. Five of these show enhanced sensitivity to ethanol, while the other five show normal sensitivity.

When Drosophila are exposed to ethanol, they first increase locomotion, then become uncoordinated and lose postural control, and finally become sedated. An apparatus that provides a readout of these behaviors is the inebriometer (Wolf and Heberlein 2003). By measuring the elution time of flies from this column, one can relate ethanol concentration in the fly to the loss of postural control. Four genes, *amnesiac*, *rutabaga*, *DCO*, and *fasiclinII*, are important for conferring normal ethanol sensitivity in Drosophila (Moore *et al.* 1998; Cheng *et al.* 2001). Each mutant gene causes an increased sensitivity to the effects of ethanol. Three, *amnesiac*, *rutabaga*, and *DCO*, are thought to act in the cAMP signaling pathway (Livingstone *et al.* 1984; Levin *et al.* 1992; Skoulakis *et al.* 1993; Feany and Quinn 1995; Moore *et al.* 1998). *fasiclinII* is involved in axon bundling, cell migration, and synapse stabilization (Cheng *et al.* 2001). All four of these genes are also necessary for at least some forms of associative learning (Keene and Waddell 2007).

Drosophila can be conditioned in several ways. Two particularly fast and robust forms of associative learning can be measured in classical olfactory and operant place learning (Tully and Quinn 1985; Wustmann and Heisenberg 1997; Putz and Heisenberg 2002). In classical olfactory learning, one of two odorants can be associated with electric shock or sugar reward. When given a choice, flies receiving this training either run away from the punishment-associated or run toward the reward-associated odorant. In operant place learning, rising temperatures are used to train a fly to avoid one-half of a small chamber (Zars *et al.* 2000b; Zars and Zars 2006). Persistent avoidance of the punishment-associated chamber half is used as a measure of place memory.

Several genes have been identified in the classical olfactory conditioning paradigm (Keene and Waddell 2007). Many of these genes are components of the cAMP signaling cascade. Furthermore, some of the mutations that alter olfactory learning also have deficits in place learning, although this has not yet been studied

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exhaustively. There are, however, differences in the genes required for the two forms of learning (Putz *et al.* 2004; Diegelmann *et al.* 2006). The *white*-ABC transporter, important for the regulation of serotonin and dopamine levels, when mutant, lowers place memory but significantly increases olfactory memory (Diegelmann *et al.* 2006; Sitaraman *et al.* 2008). Second, different mutant alleles of the S6KII gene can selectively alter memory performance in the two different tests. In contrast to the ethanol-sensitive mutants, the majority of which have memory deficits, only a few of the learning mutations have been tested for ethanol sensitivity.

To determine whether learning mechanisms are universally related to processes supporting ethanol-influenced behaviors, we tested the learning abilities of 10 new *P*-element insertion lines. Five of these lines show normal ethanol response and 5 show increased ethanol sensitivity in the inebriometer. Flies from these strains were tested in both the place-learning paradigm and a classical olfactory conditioning test. With this approach we could test the relationship between the mechanisms that support learning and behavioral resistance to ethanol. We find that in this set of genes, there is only a modest relationship between ethanol sensitivity and olfactory learning functions. We did not detect a relationship between ethanol sensitivity and place memory. Five novel loci are implicated in ethanol-influenced behaviors. Two new genes are important for learning.

MATERIALS AND METHODS

Flies: Flies were raised on cornmeal-based media in a light- and humidity-controlled chamber (12:12-hr light:dark cycle, 60% relative humidity). Flies used for behavioral experiments were <7 days old and were never anesthetized. To control for genetic background, all potential mutant lines were outcrossed to a *white*-mutant (w^{1118}) wild-type Berlin background for five or six generations. For the learning experiments, the X chromosomes were then replaced using balancer chromosomes that were themselves in a wild-type Berlin background. All lines were additionally outcrossed to a w^{1118} wild-type Canton-S background for six generations, followed by X chromosome replacement in the learning experiments with a wild-type version of this chromosome.

Behavior: The sensitivity of flies to ethanol was tested in the inebriometer as described (Moore *et al.* 1998). Briefly, groups of flies were added to the inebriometer, which was held at constant temperature and pressure to ensure a consistent ethanol vapor concentration. Flies eluting from this column after loss of postural control were counted and used to determine the mean elution time (MET).

Flies were conditioned in two paradigms. In the heat box (Wustmann *et al.* 1996), individual flies were trained to avoid part of a small chamber by associating one-half with a temperature that they typically avoid (41°). The contrasting temperature was 24°. Flies were trained for 20 min (Diegelmann *et al.* 2006). Memory was tested for 3 min directly following conditioning, when the danger of high temperatures was removed. A performance index (PI) is calculated by subtracting the time in the 41°-associated half from the time in the cooler half, all divided by the total time. Control experiments tested the ability of wild-type and potentially mutant flies to sense and avoid a high-temperature source (Zars 2001). In this test, one-half of the chamber is heated to the same temperature as that used for conditioning while the other half of the chamber is kept at 24°, a temperature that flies normally prefer. A PI is similarly calculated for this thermosensitivity test. In both the place-learning and the thermosensitivity tests, an approximately equal number of experiments paired the left or the right chamber half with the higher temperature. Because tests for normality give mixed results, nonparametric statistics were used (Putz and Heisenberg 2002). Classical olfactory conditioning paired one of two odorants (4-methylcyclohexanol and octanol) with electric shock (100 V) (Tully and Quinn 1985). Memory tests were performed 3 min after training (unless otherwise stated), where changed olfactory preferences were tested in a t-maze. Control experiments measured flies' avoidance of the odorants or shock used in the conditioning experiment. That is, an odorant at the same concentration used in the conditioning experiments was presented in one arm of the t-maze. The other arm of the t-maze had ambient air. In the shock test, two shock tubes were placed at the t-maze choice point and one of these was pulsed with 100-V electric shocks. In both control experiments, flies were allowed to choose between the two tubes for 60 sec (the same amount of decision time used in the learning experiments). A PI was calculated for the learning and control experiments and multiplied by 100, as is the tradition for this assay. This scale ranges from −100 to 100, with 0 indicating no memory or avoidance behavior. This was calculated by subtracting the number of flies choosing the shock-associated odorant from the number of flies choosing the non-shock-associated odorant, divided by the total number of flies in a "half test." An average PI was calculated from a pair of half-test PIs, where each half came from conditioning of one of the two odors. Tests for significant differences used a parametric ANOVA with Newman–Keuls *post hoc* tests when warranted (Zars *et al.* 2000a).

Molecular biology: The insertion site of the pGAwB *P* element in each of the new fly lines was determined by inverse PCR and sequencing (Dalby *et al.* 1995). Inverse PCR followed genomic DNA restriction digest with *Hpa*II, ligation, and PCR using primers GTC CGC ACA CAA CCT TTC C/GAG GAT GAC ATG TCG GAT GG or primers CGG GAC CAC CTT ATG TTA TTT C/CTG AGT GAG ACA GCG ATA TG. The sequenced PCR products were compared to the Drosophila genome to identify the *P*-element location. The insertion site was confirmed using PCR with primers specific for the sequence on either side of the *P* element and the adjacent genome. Furthermore, after outcrossing the *P*-element insertion was reconfirmed, using the gene-specific primer pairs. This proved both the location of the insertion and that the outcrossed flies are homozygous for the insertion. We can detect a nonmutant chromosome at a contaminant ratio of at least 1:50.

Quantitative RT–PCR: The relative levels of *tribbles* and *CG2185* were determined by quantitative (q)RT–PCR. Total RNA was extracted from two or three samples of ~200 mixed male and female fly heads using the Trizol reagent (Invitrogen, San Diego). The RNA was collected from the 3-54 and 10-110 strains. mRNA was purified using an mRNA minipurification kit (QIAGEN, Valencia, CA). A total of 100 ng of mRNA were used as a template in at least two reverse transcriptase cDNA reactions from each sample (Superscript III, Invitrogen). Three genes were amplified in an Applied Biosystems (Foster City, CA) 7500 Fast Real-Time PCR system. These were *tribbles* (primers ATT CGT TCC GTG TAA TCC TT and CAA CCT CAT CAC CGT CAT AC), *CG2185* (primers TGC GAG CGG ATA GTT CAT TCC T and TCT GCT GAT CAC ACC ATC GTC A), and *rp49* (primers AGC GCA CCA AGC ACT TCA

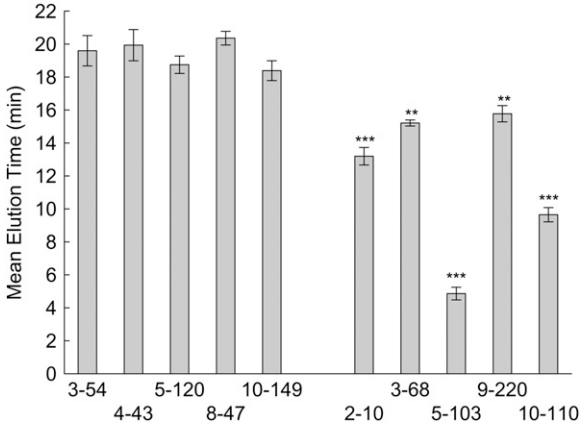

FIGURE 1.—Ten new pGAL4 insertion lines and their ethanol responsiveness. Five lines had a mean elution time of ~19 min. This is in contrast to 5 mutant lines that had significantly reduced mean elution times [$F(9, 58) = 61.3$, $P < 0.0001$; differences after Newman–Keuls *post hoc* test of the mutant lines compared to 10-149, the lowest normal performance line, are shown: $^{**}P < 0.01$, $^{***}P < 0.001$]. The values represent means and error bars are SEMs.

TCC GCC A and GCG CAC GTT GTG CAC CAG GAA CTT C). The efficiency of amplification was determined for each gene using a series of twofold cDNA dilutions and used in calculating relative concentrations of *tribbles* and *CG2185* (PFAFFL 2001). The efficiencies for amplification of *tribbles*, *CG2185*, and *rp49* were 1.93, 1.94, and 1.90. A two-tailed *t*-test with the assumption of unequal variance was used to test for significant differences between the fold differences in expression and the null hypothesis that there would be no difference in expression levels between the genotypes (a fold difference value of 1).

RESULTS

Our strategy for testing the ethanol response/learning mechanisms was to first identify new ethanol-sensitive mutant lines and an equal number of lines from the same mutagenesis that had essentially normal ethanol response. From ~1000 newly generated *P*-element insertion lines that were screened for ethanol sensitivity (D. GUARNIERI and U. HEBERLEIN, unpublished observation), we characterized the memory phenotypes of 5 lines that are normal and 5 lines that are sensitive to ethanol (Figure 1). The 5 sensitive lines that were tested for memory phenotypes were chosen because they have strong to moderate sensitivities to ethanol (~25–75% of normal levels), good viability, and high fecundity and appear rather normal in food vials. The 5 control lines were chosen because they also appear generally healthy and because their ethanol sensitivity was similar to the average mean elution time of the lines screened.

We did memory tests of mutant and normal ethanol-sensitivity lines in groups of three or four together with an appropriate wild-type strain. The constraint of testing different genotypes in parallel and the labor-intensive nature of memory testing required this bundling of experiments. The first tests were done in a wild-type Canton-S (CS) genetic background because these flies typically perform better than wild-type Berlin flies in olfactory learning, as has been seen for other wild-type strains (DUDAI 1977). Thus, subtle differences between mutant and control lines might be more evident. Furthermore, learning tests were done blind with respect to ethanol-sensitivity phenotypes.

The first memory tests were done in the heat-box place-learning paradigm. Flies were trained for 20 min and tested for place memory immediately afterward for 3 min. The memory scores are presented in Figure 2. There was some variation in memory performance of the different mutant lines, but only the line 3-54 showed a significantly reduced memory score compared to wild-type CS flies' performance levels. Thus, the only line to show a significant effect here was normal in its ethanol sensitivity.

We next tested whether the 3-54 mutant place-learning phenotype could be a consequence of an inability to sense or avoid the high temperatures used in this paradigm. Wild-type CS and 3-54 mutant flies were allowed to choose between chamber halves that were either 24° or 41°. We found no differences in the ability of CS or 3-54 flies to avoid a high-temperature source used in the learning assay (Figure 2D).

Wild-type, mutant, and control flies were next tested in classical olfactory conditioning. We reasoned that we might find different lines defective in the two types of learning as there are differences in the neural systems, biogenic amines, and genes that are critical between the paradigms (ZARS *et al.* 2000a,b; SCHWAERZEL *et al.* 2003; PUTZ *et al.* 2004; SITARAMAN *et al.* 2008). In this case, one of two odorants is paired with electric shock and flies are allowed to choose between the shock-associated and nonassociated odorants. We again tested three or four groups of mutant flies and wild-type CS flies in parallel (Figure 3). Interestingly, memory scores were significantly altered in 2 of the 10 ethanol lines. The line 3-54, previously found to reduce place-memory performance, has a significantly higher memory score than that of wild-type CS flies (~120% of wild-type levels). In contrast, 10-110 flies have a memory level at ~60% that of wild type.

To seek the first clues as to the time domain in which the 3-54 mutant memory levels are abnormally high, we tested memory at four time points between 3 and 180 min after training. Interestingly, the 3-54 mutant memory remains elevated relative to wild-type performance even 3 hr after training. Furthermore, the slopes of the memory decay curves in the two lines are roughly parallel. This suggests that the 3-54 phenotype is one that alters an early process in memory formation.

The 3-54, 10-110, and wild-type CS flies were next tested for their ability to sense and avoid the odors and electric shock used for conditioning experiments. The 3-54 and 10-110 mutant flies did not have significant differences from the wild-type CS flies' olfactory avoid-

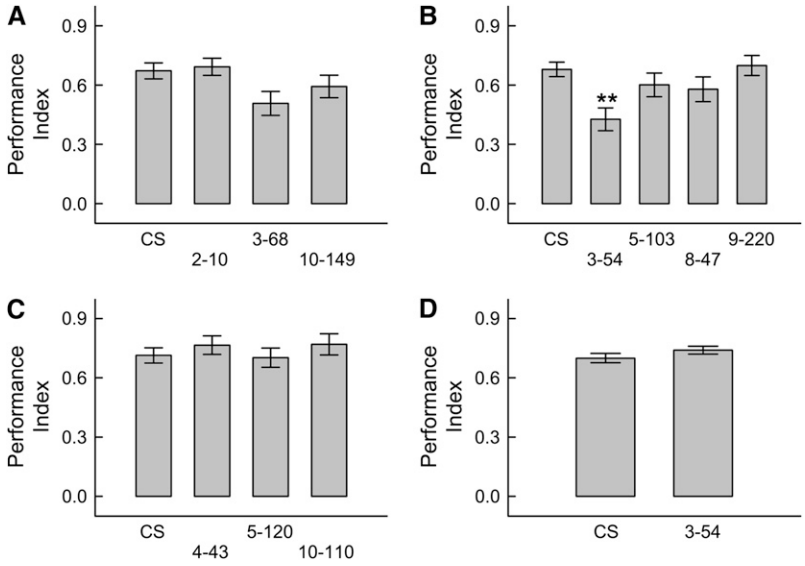

FIGURE 2.—Only the 3-54 mutant flies have a significant change in place-memory performance. (A) There were no significant differences in the memory scores of these groups of flies [H (3, $N = 655$) = 10.9, $P = 0.012$; *post hoc* tests with multiple comparisons found no significant differences among these strains, although 3-68 approaches significance ($P = 0.06$)]. (B) The 3-54 insertion line flies' memory performance was reduced compared to control and all other insertion line genotypes tested [H (4, $N = 697$) = 24.2, $P = 0.0001$]. Significant differences after multiple comparisons are presented, 3-54 with each group (**$P < 0.01$). (C) No significant differences were found in this set of lines [H (3, $N = 585$) = 4.3, $P = 0.23$]. (D) Tests for the ability of 3-54 and wild-type CS flies to sense and avoid a 41° temperature source did not find significant differences between genotypes (U-test: $Z = 1.02$, $P = 0.31$, $N = 211$). The values represent means and error bars are SEMs.

ance behavior (Table 1). In contrast, the shock reactivity of 10-110 flies was reduced compared to wild-type CS levels. Thus, the high 3-54 olfactory memory is not a consequence of changes in sensory acuity. However, the 10-110 memory phenotype may be influenced by lower shock reactivity.

To gain confidence that the 3-54 and 10-110 mutations cause the memory defects that we measured, we also tested these mutant lines in a wild-type Berlin background. When tested in the heat box, 3-54 flies had a significantly reduced memory performance level compared to wild-type Berlin flies (Figure 4). The magnitude of the memory deficit was smaller in the Berlin background than in the Canton-S background, however. Olfactory memory in 3-54 flies is ~130% of wild-type Berlin levels. Consistent with the Canton-S comparisons, 10-110 flies have normal place memory but strongly reduced olfactory memory. Control experiments for olfactory avoidance and shock reactivity yielded similar results to those found for these lines in the Canton-S genetic background (Table 1). Furthermore, the high-temperature avoidance behavior was not different between Berlin (PI = 0.49 ± 0.05) and 3-54 (PI = 0.54 ± 0.05) flies ($U = 7149$, $Z = 1.26$, $P = 0.21$, $N = 251$).

We next tested whether there was a correlation between ethanol sensitivity and memory formation in the mutant and control lines that we tested. Mean elution times for the 10 strains ranged from ~5 min to 20 min and memory scores from ~40 to 85. There is no relationship identified in place-memory scores and ethanol sensitivity in the 10 lines studied here (Figure 5). In contrast, we found a trend for a correlation between ethanol sensitivity and olfactory memory, although this did not reach statistical significance. Tests

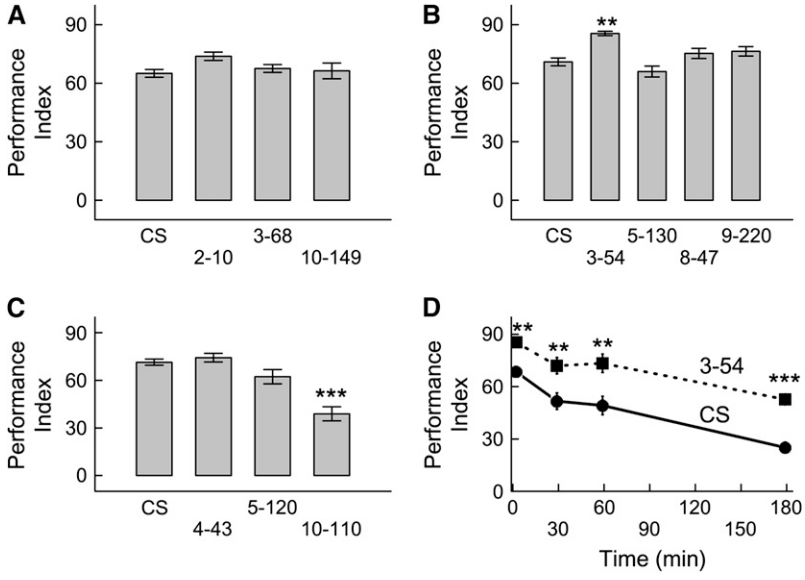

FIGURE 3.—The 3-54 insertion line flies perform better than, but 10-110 flies perform worse than, all other groups tested. (A) There were no significant differences found between these groups of flies (ANOVA: $F = 2.07$, $P = 0.14$). (B) 3-54 flies' memory performance was increased compared to control and all other insertion line genotypes tested (ANOVA: $F = 10.29$, $P < 0.0001$). Significant differences after multiple comparisons are presented, 3-54 flies with each group (**$P < 0.01$). (C) The 10-110 insertion line flies' memory performance was strongly reduced (ANOVA: $F = 20.11$, $P < 0.0001$). Significant differences after multiple comparisons are presented, 10-110 with each of the three groups of flies shown (***$P < 0.001$). (D) 3-54 insertion flies perform significantly better than wild-type CS tested at four time points following training (ANOVA: 3 min, $F = 88.89$, $P < 0.01$; 30 min, $F = 9.26$, $P < 0.01$; 60 min, $F = 10.54$, $P < 0.01$; 180 min, $F = 39.63$, $P < 0.001$). Significant differences are presented. $N = 6$ for each genotype and time point. The values represent means and error bars are SEMs.

TABLE 1

Control behaviors in wild-type and mutant fly lines

Genotype	Shock reactivity	MCH avoidance	OCT avoidance
CS	84.7 ± 1.4	21.4 ± 3.8	29.1 ± 3.4
3-54^{CS}	86.3 ± 2.4	16.8 ± 5.8	29.2 ± 5.6
10-110^{CS}	67.8 ± 3.4***	15.4 ± 6.5	22.3 ± 2.7
Berlin^{1}	88.9 ± 2.4	22.7 ± 4.7	33.9 ± 4.8
3-54^{B}	85.0 ± 3.9	13.8 ± 6.6	43.7 ± 5.0
Berlin^{2}	83.2 ± 4.6	12.6 ± 7.5	34.3 ± 6.8
10-110^{B}	59.6 ± 5.4***	19.0 ± 3.0	17.6 ± 4.5

Control behaviors were measured in wild-type Canton S (CS), wild-type Berlin, 3-54, and 10-110 flies. The 3-54 and 10-110 mutant lines were in either the CS or the Berlin genetic backgrounds (labeled with the CS or the B superscript). A significant difference was found in shock reactivity of CS, 3-54^{CS}, and 10-110^{CS} flies [F (2, 21) = 19.2, $P < 0.001$; significant difference between CS and 10-110^{CS} after Newman-Keuls *post hoc* test is shown, ***$P < 0.001$]. There was no significant difference between genotypes for avoidance of the odors MCH [$F(2, 21) = 0.44$, $P = 0.65$] and OCT [$F(2, 21) = 0.83$, $P = 0.45$]. There were significant differences in shock reactivity of flies in the Berlin background [$F(3, 20) = 9.8$, $P < 0.001$; significant difference between Berlin^{2} and 10-110^{B} after Newman-Keuls *post hoc* test is shown, ***$P < 0.001$]. OCT avoidance behavior was significantly different between genotypes [$F(3, 20) = 4.05$, $P = 0.02$], but relied on differences between 3-54^{B} and 10-110^{B}. There were no significant differences between genotypes in the Berlin background for MCH avoidance [$F(3, 20) = 0.68$, $P = 0.58$]. Values are means ± SEMs.

with additional lines might reveal a more consistent relationship between these two traits.

We were interested in the genes that were altered in the 10 lines we tested. Inverse PCR, sequencing, and comparison to the genome identified the *P*-element insertion site of all 10 lines (Table 2). To confirm the insertion sites, we designed primers that anneal within the genomic sequence and the *P*-element termini. We identified unique PCR products for each of these lines, confirming the insertion sites. Three genes are of special interest here. The 3-54 insertion site is ~250 bp upstream of the first exon of the *tribbles* gene. This gene seems to function in the regulated stability of certain proteins (see DISCUSSION). Furthermore, 10-110 has an insertion in the first exon of *CG2185*. And finally, the 8-47 insertion is within the first intron of the "A" transcript of *pathetic*, an amino acid transporter. While this gene has been shown to be critical for growth control and interacts with the insulin-signaling cascade (GOBERDHAN *et al.* 2005), this mutant allele does not have a phenotype in ethanol sensitivity or learning.

In our first attempts to determine whether the genes closest to the *P*-element insertion sites are responsible for the changes in learned behavior, we performed qRT–PCR experiments. We concentrated first on the *tribbles* and *CG2185* genes as flies with *P* elements close to these genes have the strongest memory phenotypes. The relative fold difference in expression levels of *tribbles* and *CG2185* was determined using mRNA isolated from 3-54 and 10-110 fly heads. The *rp49* gene was used as a control gene to compensate for any potential differences between strains in cDNA synthesis. Using qRT–PCR results from each of these genes in 3-54 and 10-110 flies, we determined the relative fold differences in expression between the two strains (PFAFFL 2001). For the *tribbles* gene, we found that 3-54 fly heads had 0.64-fold of the 10-110 fly head levels (Table 3). This corresponds to an ~1.5-fold lower expression level of *tribbles* in 3-54 compared to 10-110 fly head mRNA. In contrast, the 10-110 flies had 0.24-fold (or a 5-fold lower level) of the 3-54 fly head *CG2185* mRNA levels (Table 3). The specific lowered levels of *tribbles* in 3-54 and *CG2185* in 10-110 fly heads suggest that these genes are critical for memory formation.

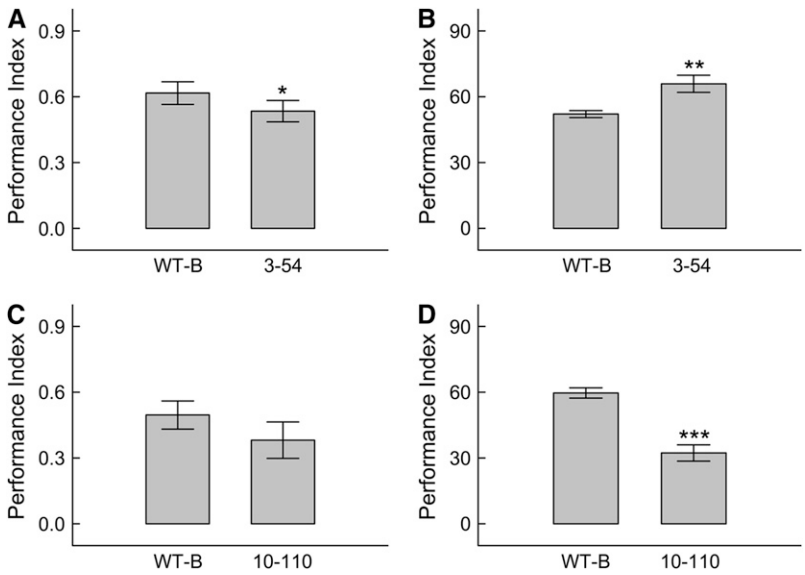

FIGURE 4.—The 3-54 and 10-110 mutations have a consistent phenotype in the wild-type Berlin (WT-B) genetic background. (A) 3-54 flies perform significantly worse than WT-B flies in place memory (*U*-test: $Z = 1.96$, $*P = 0.049$, $N = 331$). (B) 3-54 flies perform significantly better than WT-B flies in olfactory classical conditioning. (ANOVA: $F = 10.69$, $**P < 0.01$, $n = 6$ for each genotype). (C) There were no significant differences between 10-110 and WT-B flies when tested for place memory (*U*-test: $Z = -0.55$, $P = 0.58$, $N = 260$). (D) 10-110 flies performed significantly worse than WT-B flies in olfactory classical conditioning (ANOVA: $F = 43.07$, $***P < 0.001$, $n = 6$ for each genotype). The values represent means and error bars are SEMs.

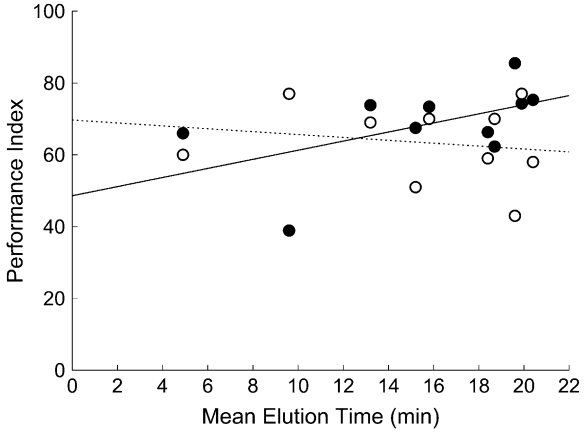

FIGURE 5.—No correlation between ethanol sensitivity and memory performance. The place-memory (open symbols) and olfactory-memory (solid symbols) scores were plotted with respect to mean elution time in the 10 lines described here. There was no correlation (dashed line) between place memory and the mean elution time ($R = 0.18$, $P = 0.6$). A weak but not significant correlation (solid line) was found between olfactory memory and the mean elution time ($R = 0.52$, $P = 0.11$).

DISCUSSION

We provide three significant results. First, we have identified five new mutations that alter the behavioral response of flies to ethanol. The genes altered in this fashion include three genes that have yet-to-be-identified cellular function, a locus that could alter either or both of two closely associated genes, and a fifth gene that alters a poly(Q) tract binding protein (YOSHIMURA *et al.* 2006). Second, we have identified two new learning mutations. These include changes in *tribbles*, altering a gene with a function in regulating protein degradation. The other learning mutation we identified, altering *CG2185*, suggests a role of this putative Ca^{2+}-binding protein in memory formation. Third, in the 10 lines tested here we find no relationship between ethanol sensitivity and place memory. Furthermore, we find only a weak nonsignificant relationship between olfactory memory and ethanol sensitivity.

Our conclusion on finding new learning genes depends on close association of phenotypes in two different genetic backgrounds and changes in gene expression. It seems likely that the gene most closely associated with the *P*-element insertion site should be the cause of the mutant phenotypes that we measured. The relative reduction of *tribbles* in 3-54 and *CG2185* in 10-110 fly heads supports this notion. For the genes not tested in this fashion, we cannot, however, rule out the possibility of a closely linked locus surviving the outcrossing scheme and this locus being important for changes in behavior.

TABLE 2

pGAL4 insertions and behavioral phenotypes

Name of insertion	Behavioral phenotypes	Associated gene	Cellular function
2-10	↑ ES	PQBP-1, 32 bp 5′ of exon 1	Poly(Q) tract binding protein (YOSHIMURA *et al.* 2006)
3-54	↓ PM, ↑ OM	*tribbles* (*trbl*), ~250 bp 5′ of exon 1	Protein degradation regulation (MATA *et al.* 2000; RORTH *et al.* 2000)
3-68	↑ ES	*CG31886*, exon 1	Unknown
4-43	Normal	Tetraspanin at 42El ~35 bp 5′ of exon 1	Ectoderm and nervous development (GRUMBLING and STRELETS 2006)
5-103	↑ ES	*CG33472*, 3′ end of predicted gene	Unknown
5-120	Normal	*scabrous* (*sca*), ~125 bp 5′ of exon 1	Development of nervous system, eyes, sensory bristles, and wings (POWELL *et al.* 2001)
8-47	Normal	*pathetic* (*path*), intron 1 of transcript A	Amino acid transporter (GOBERDHAN *et al.* 2005)
9-220	↑ ES	Between (1) *l(2)0128*, ~100 bp 5′ of exon 1 and (2) *putative homeodomain transcription factor* (*phtf*), ~1 Kbp 5′ of exon 1	 *l(2)01289*: electron transport (GRUMBLING and STRELETS 2006) *phtf*: putative homeodomain transcription factor (MANUEL *et al.* 2000)
10-110	↑ ES, ↓ OM	*CG2185*, exon 1	Ca^{2+}-binding protein (GRUMBLING and STRELETS 2006)
10-149	Normal	Between (1) *Glial Lazarillo* (*GLaz*), ~1.5 Kbp 5′ of exon 1 and (2) *Serine palmitoyltransferase subunit I* (*Spt-I*), ~450 bp 5′ of exon 1	 *GLaz*: lipocalin gene product (SANCHEZ *et al.* 2000) *Spt-I*: lipid metabolism (GRUMBLING and STRELETS 2006)

ES, ethanol sensitivity; PM, place memory; OM, olfactory memory.

TABLE 3

Relative levels of *tribbles* and *CG2185* in 3-54 and 10-110 fly head mRNA

Gene	Fold expression level (mean ± SEM)	*P*-value
tribbles	0.64 ± 0.02	1.4×10^{-8}
CG2185	0.24 ± 0.03	3.3×10^{-9}

Proof of gene function obviously still depends on tests of multiple alleles and transgenic approaches.

We speculate here on how *tribbles* could be regulating memory formation. The effect of *tribbles* could be one that influences the development of the nervous system, regulates a cellular process during learning, or both. The previously identified molecular functions of *tribbles* can support several models. Evidence to date indicates the *tribbles* product is a pseudokinase that may serve to target proteins to a ubiquitin ligase and degradation (Mata *et al.* 2000; Rorth *et al.* 2000; Qi *et al.* 2006). Known target proteins in Drosophila include *string/cdc25* (a regulator of the cell cycle) and *slow-borders* (a c/ebp protein). In mammalian studies, *tribbles* homologous genes have been shown to regulate Akt/PKB and Acetyl-CoA carboxylase protein levels (Du *et al.* 2003; Qi *et al.* 2006). These genes are excellent candidates for regulating either the developing or adult CNS to influence memory formation and are the target of ongoing investigation.

The *CG2185* gene, which we name here, ***e**thanol sensitive* with ***l**ow **m**emor**y*** (*elm*), has an EF-hand domain that might indicate this protein binds calcium (Grumbling and Strelets 2006). Furthermore, *elm* is most similar to calcineurin B subunits in other insects (Nene *et al.* 2007) and to the P22 protein in vertebrate animals (Barroso *et al.* 1996). The P22 protein has a role in Ca^{2+}-dependent regulation of membrane trafficking and calcineurin activity (Lin *et al.* 1999; Andrade *et al.* 2004). Whether *elm* has a similar function within the nervous system to influence behavior will be the focus of future study. Furthermore, naming of the other ethanol-sensitive mutations will await additional phenotypic characterization. For the other genes we examined that did not have a learning or ethanol-sensitivity phenotype, naturally, we do not rule out their role in behavior. We conclude only that the alleles tested did not have an effect on the behaviors measured.

With the 10 lines tested here and previous descriptions, there are four mutant patterns with respect to ethanol sensitivity, place memory, and olfactory learning. These patterns might suggest mechanisms of action for these genes:

i. We found four ethanol-sensitive mutations that did not alter memory formation in either paradigm. This is a novel class and should provide insights into specific mechanisms of drug effects on behavior.
ii. In the case of *elm*, we found sensitivity to ethanol and strong reduction in olfactory memory. That one can mutate olfactory learning but leave place memory intact has been seen in specific alleles of the S6KII gene (Putz *et al.* 2004). Perhaps the *elm* product has a function related to S6KII activity.
iii. The *tribbles* gene provides a suite of behavioral changes similar to that found by mutation of the *white*-ABC transporter. In both cases place memory can be strongly reduced but olfactory memory is significantly elevated (Diegelmann *et al.* 2006). As both serotonin and dopamine levels are strongly altered by mutation of the *white*-ABC transporter (Sitaraman *et al.* 2008), and these amines play a role in place and olfactory memory (Schwaerzel *et al.* 2003; Sitaraman *et al.* 2008), perhaps the *tribbles* gene alters the biogenic amine systems or their effects to alter memory. Mutation of the *white* gene has effects on several other behaviors, including courtship, anesthesia resistance, and aggression (Zhang and Odenwald 1995; Campbell and Nash 2001; Hoyer *et al.* 2008). Strikingly, a null mutant allele of the *white* gene does not alter ethanol sensitivity (D. Guarnieri and U. Heberlein, unpublished observation), consistent with the *tribbles* phenotypes.
iv. We have previously found that mutations of genes in the cAMP signaling cascade increase sensitivity to ethanol and reduce memory in both paradigms (Levin *et al.* 1992; Feany and Quinn 1995; Moore *et al.* 1998; Zars *et al.* 2000a,b). Thus, in this study we found examples of mutations that alter the three other possibilities for mutant effects in ethanol response and memory.

Although we have doubled the number of ethanol-sensitive mutations that have been examined for learning deficits, our conclusions are still limited to a handful of genes. In another screen, several genes implicated in long-term memory (Dubnau *et al.* 2003) are also important for ethanol sensitivity (K. Berger and U. Heberlein, unpublished data). In this group of flies, however, an overall correlation between ethanol sensitivity and memory phenotype was also not significant. Furthermore, the memories tested involve only relatively simple aversive conditioning paradigms. Thus, a more significant correlation between ethanol response and memory formation might be found by testing more complex aversive and appetitive conditioning protocols (Tempel *et al.* 1983; Schwaerzel *et al.* 2002; Zars and Zars 2006; Sitaraman *et al.* 2007). Nevertheless, our behavior genetic data suggest there are several mechanisms of resistance to ethanol and memory formation. Complexity at the level of genes, neural circuits, and behavior is to be expected between these two processes.

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