Neurobiology of Learning and Memory 144 (2017) 68–76

Contents lists available at ScienceDirect

Neurobiology of Learning and Memory

journal homepage: www.elsevier.com/locate/ynlme

The *Drosophila melanogaster tribbles pseudokinase* is necessary for proper memory formation

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ARTICLE INFO

Article history:
Received 17 April 2017
Revised 27 June 2017
Accepted 28 June 2017
Available online 29 June 2017

Keywords:
Tribbles
Pseudokinase
Learning
Memory
Behavior

ABSTRACT

The *tribbles (trbl) pseudokinases* play important roles in signaling and physiology in multiple contexts, ranging from innate immunity to cancer, suggesting fundamental cellular functions for the *trbls*' gene products. Despite expression of the *trbl pseudokinases* in the nervous systems of invertebrate and vertebrate animals, and evidence that they have a function within mouse and human dopamine neurons, there is no clear case for a function of a Trbl protein that influences behavior. Indeed, the first and only evidence for this type of function comes from *Drosophila melanogaster*, where a mutation of the single *trbl* gene was identified in a genetic screen for short-term memory mutant flies. The current study tested flies containing multiple *trbl* mutant alleles and potential transgenic rescue in both operant place memory and classical olfactory memory paradigms. Genetic complementation tests and transgenic rescue of memory phenotypes in both paradigms show that the *D. melanogaster trbl pseudokinase* is essential for proper memory formation. Expression analysis with a polyclonal antiserum against Trbl shows that the protein is expressed widely in the fly brain, with higher expression in the cellular rind than the neuropil. Rescue of the behavioral phenotype with transgenic expression indicates the *trbl* function can be localized to a subset of the nervous system. Thus, we provide the first compelling case for the function of a *trbl pseudokinase* in the regulation of behavior.

1. Introduction

The *tribbles* (*trbl*) family of pseudokinases play multiple critical roles in physiology and disease (Eyers, Keeshan, & Kannan, 2017). These pseudokinases are thought to both link substrate binding to specific protein stability by recruiting ubiquitin ligases and as regulators of MAPK and AKT/FOXO - signaling (Eyers et al., 2017). There is one version of the *trbl pseudokinase* in *Drosophila melanogaster* and *Caenorhabditis elegans* (Kim et al., 2016; Mata, Curado, Ephrussi, & Rorth, 2000; Pujol et al., 2008). Mouse and man have three, named *trbl 1, 2,* and *3* (Boudeau, Miranda-Saavedra, Barton, & Alessi, 2006; Eyers et al., 2017). There is wide-spread expression of the three mammalian *trbl* products in brain and the *D. melanogaster trbl* gene is expressed in the developing nervous system (Aime et al., 2015; Fisher et al., 2012; Ord et al., 2014). Nevertheless, there is little known about how *trbl* influences brain function. The vertebrate *trbl 3* has been implicated in Parkinson's disease as a cell death promoter in dopaminergic neurons (Aime et al., 2015). However, knock-out of *trbl 3* in the mouse has no effect on feeding behavior, or learning and memory in three different paradigms (Ord et al., 2014). Behavioral tests on *trbl 1* or *2* have not been reported (Lin et al., 2016; Satoh et al., 2013). Thus far the only evidence for a function of *trbl* in regulating behavior is from a genetic screen to identify short-term memory fly mutants (LaFerriere et al., 2008; Masoner et al., 2013). Whether the *Drosophila trbl* gene, as a key example of *trbl pseudokinase* function, plays a definitive role in memory is the focus of this study.

Memory is readily examined in *D. melanogaster*. Operant place learning tests individual flies for the ability to avoid part of a simple chamber associated with aversive high temperatures (Ostrowski & Zars, 2014; Wustmann, Rein, Wolf, & Heisenberg, 1996; Zars, 2010). That is, flies are conditioned to avoid one of two halves of a narrow chamber. Flies can be trained in minutes, memory lasts for up to 2 h (Diegelmann, Zars, & Zars, 2006; Ostrowski, Kahsai, Kramer, Knutson, & Zars, 2015; Putz & Heisenberg, 2002). Flies show a memory by persistent avoidance of the chamber half associated with high temperature. A second well established learning paradigm is olfactory classical conditioning (Guven-Ozkan & Davis, 2014; Tully & Quinn, 1985; Zars, Fischer, Schulz, & Heisenberg, 2000). In this case, flies are

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http://dx.doi.org/10.1016/j.nlm.2017.06.006
1074-7427/

presented with an odor that is paired in time with electric shocks or sugar reward. A second odor is also presented to flies but not associated with the shock or sugar. Flies can again be trained in minutes, and memory can be readily tested for hours to days after training. Flies show a memory in a T-maze choice point by avoiding an odor previously associated with electric shock, or approaching an odor previously associated with sugar.

The role of the *trbl* gene in memory formation was investigated here. First, complementation tests using flies with multiple mutant alleles were tested with both operant place conditioning and aversive olfactory conditioning. Second, to gain insights into where in the nervous system *trbl* is expressed, the expression pattern of the Trbl protein was examined. Finally, spatially restricted expression of the wild-type *trbl* transgene in an otherwise mutant *trbl* fly was used to rescue the mutant phenotype in both the operant place conditioning paradigm and the olfactory aversive conditioning paradigm. The results show that the *D. melanogaster trbl* gene can influence nervous system function in behavior and is required in a specific subset of the nervous system for normal memory formation.

2. Materials and methods

2.1. Fly rearing

D. melanogaster were raised on cornmeal-based media in a light, temperature, and humidity controlled chamber. Flies were kept on a 12-h light-dark cycle and held at 24 °C and 60% relative humidity. Flies used for behavioral experiments were between three and six days old and were never anesthetized.

2.2. Fly stocks and crosses

To control for genetic background, all potential mutant lines were out-crossed to a *white*-mutant (w^{1118}) wild-type Canton S (CS) background for six generations. The X-chromosomes were then replaced using balancer chromosomes that were themselves in a wild-type CS background. Mutant *trbl* alleles that were tested were $trbl^{3\text{-}54}$, $trbl^{1119}$ and $trbl^{3519}$ (LaFerriere et al., 2008; Rorth, Szabo, & Texido, 2000). The $trbl^{1119}$ and $trbl^{3519}$ are hypomorphic alleles (Masoner et al., 2013). Based on complementation tests $trbl^{3\text{-}54}$ is also likely a loss-of-function allele (below). The UAS-*trbl* transgene was provided by Pernille Rorth (Rorth et al., 2000). The *c155*Gal4 driver is an enhancer trap in the *elav* gene, *Df(3L)ri-79c* is a deficiency at the *trbl* locus; both were provided by the Bloomington *Drosophila* Stock Center (Juergens, Wieschaus, Nuesslein-Volhard, & Kluding, 1984; Lin & Goodman, 1994).

2.3. Operant place conditioning in the heat box

Operant place conditioning was performed in the heat-box. In this apparatus, single flies are allowed to walk in a dark narrow chamber (34 × 1 × 3 mm) that is lined both top and bottom with Peltier elements (Wustmann et al., 1996). There is no light source with which the flies might see. The position of the fly was monitored at 10 Hz, temperature within the box was controlled by the Peltier elements (Zars, Wolf, Davis, & Heisenberg, 2000). One half of the experiments associate high temperatures with the front half of the chamber. The other half of the experiments associates high temperatures with the back half. The temperature of 24 °C was used for the non-punished temperature as flies have a strong preference for this temperature over both higher and lower temperatures (Sayeed & Benzer, 1996; Zars, 2001) and 41 °C was used as the high temperature aversive reinforcement as this is a temperature they avoid. Conditioning consists of three phases. First flies were allowed to walk in the chamber for 30 s during a pre-test phase where the chamber is held at 24 °C. Conditioning immediately follows the pre-test. Here flies are trained for twenty minutes to avoid the punished half of the chamber. When a fly enters the punished half of the chamber by crossing an invisible midline the whole chamber heats to 41 °C and when it enters the unpunished half the whole chamber cools to 24 °C. In this case, place memory was measured directly after training for three minutes. This test provides a single measure of a memory (Putz & Heisenberg, 2002; Sitaraman, Zars, & Zars, 2007; Zars & Zars, 2006). During the memory test the chamber temperature is held at 24 °C.

2.4. Thermosensitivity

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 was heated to the same temperature as that used for conditioning while the other half of the chamber is kept at 24 °C, a temperature that flies normally prefer. This provides a step-gradient for the flies, in which one half of the chamber is at a higher temperature than the other. This is in contrast to the conditioning experiments, in which the temperature of the whole chamber rises and falls depending on whether the fly moves to the front or rear of the chamber. An equal number of experiments paired the front or back chamber-half with the higher temperature.

2.5. Aversive olfactory conditioning

Aversive olfactory conditioning was performed by pairing one of two odorants (4-methylcyclohexanol or octanol) with 100 V of electric shock (Tully & Quinn, 1985). This was done under dim red light at between 85 and 95% relative humidity. 100–150 flies are trapped in a copper wire-lined tube where they are presented with either of the two odors. The first odor presentation was paired with electric shock every five seconds for 1.2 s over one minute, followed by a one-minute rest period with a clean airstream. The second odor was then presented with no shock. Memory tests were performed 3 min after training. Altered olfactory preferences were tested in a T-maze. Flies were allowed one minute to choose between two arms one containing the odor associated with shock and the other containing the non-shock associated odor. This choice was made in complete darkness.

2.6. Shock sensitivity and odor avoidance

Control experiments for olfactory conditioning measured flies' sensitivity to the odors and shock used in the conditioning experiment. For odor sensitivity tests, naïve flies were given a choice at the T-maze choice point between entering an arm containing an odor at the same concentration used in the conditioning experiments and entering the other arm which had air from the room. 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 every five seconds for 1.2 s over one minute as in the conditioning experiment. In both odor and shock control experiments, flies were allowed to choose for 1 min (the amount of time used in the conditioning experiments). The number of flies in both tubes were counted.

2.7. Performance index

A performance index is used to quantify fly behavior and memory in each paradigm and is calculated the same way for conditioning and control experiments.

A performance index (PI) for operant place memory is calculated as the time in the punishment-associated chamber half subtracted from the time in the non-punishment-associated chamber half, all divided by the total time in a training session (Wustmann et al., 1996). The maximum performance index is 1.0 and indicates perfect avoidance of the chamber-half associated with high temperature. A performance index of zero indicates preference for neither chamber half.

For olfactory conditioning a performance index is quantified as the number of flies that approach the non-shock associated odor minus the number of flies that approach the shock associated odor all divided by the total number of flies in a half-test. A complete olfactory experiment PI is calculated as the average from a pair of half-test PIs, where each half came from using one of the two odors as the punished odor in one conditioning experiment. Memory or avoidance performance is represented on a scale from −1 to 1, with 0 indicating no memory.

2.8. Behavior statistics

Place memory and thermosensitivity scores were tested using non-parametric statistics (Putz, Bertolucci, Raabe, Zars, & Heisenberg, 2004; Putz & Heisenberg, 2002). That is, when multiple groups were compared a non-parametric Kruskal Wallis ANOVA was used. Tests for significant differences in olfactory conditioning and control experiments used a parametric ANOVA with Neuwmann-Keuls post-hoc tests (Zars, Fischer, et al., 2000). Statistica 8.0 software was used for all tests.

2.9. Immunohistochemical analysis of the fly brain

Drosophila brains were extracted from 3 to 5 day old adults (Rein, Zockler, Mader, Grubel, & Heisenberg, 2002). First the proboscis and then the eyes were removed with fine forceps while the whole fly was pinned at the thorax and abdomen in Ringer's solution (130 mM NaCl, 0.7 mM KH_2PO_4, 0.35 mM Na_2HPO_4, 18 mM $MgCl_2$ and 4.7 mM KCl). The brains were then fixed in 2% formaldehyde in PAT (100 ml 1XPBS, 1% BSA and 0.5% Triton X-100) solution overnight at 4 °C. Brains used to visualize GFP expression were blocked for two hours with 3% normal goat serum in PAT solution at room temperature followed by overnight incubation with the two primary antibodies, anti-5HT (1:10 in PAT; Biomeda) and anti-GFP (1:20 in PAT; Sigma) at 4 °C. Secondary antibodies Alexa 488 goat anti rabbit (1:100 in PAT; Invitrogen) and Alexa 647 goat anti-mouse (1:50 in PAT; Invitrogen) were added together and incubated overnight at 4 °C.

Brains immunolabeled with the Trbl antiserum: Fixed brains were blocked using Hen-Block (1:10 in PAT; Aves Lab) at room temperature for one and a half hours followed by overnight incubation at 4 °C in anti-Trbl (1:1000 in PAT). The affinity-purified chicken polyclonal anti-Trbl antibody was raised against a peptide corresponding to amino acids CZDKHEYEDIGVEPLDYTR of the *Drosophila* Trbl protein (Aves Lab, Tigard, OR). The brains were also incubated with anti-Bruchpilot (Brp) (Rein et al., 2002), a synaptic active zone marker, (1:100 in PAT) for 2 h at room temperature followed by incubation with secondary antibodies FITC anti-chick (1:500) and Alexa 647 goat anti-mouse (1:100; Invitrogen) both in PAT. All antibody incubations were followed by three 10 min washes with PAT.

Brains were mounted in Vectashield reagent (Vector Laboratories) (1:3 Ringers: Vectashield) in a narrow well made from four coverslips and fingernail polish. The whole mount brains were visualized using an LSM 510 NLO confocal microscope with 20X objective. Images were examined using the LSM examiner software.

3. Results

Complementation tests were used to determine whether the mutant phenotype observed in the insertion line $trbl^{3\text{-}54}$ is indeed due to a defect in the *trbl* gene (LaFerriere et al., 2008). Multiple insertion alleles of *trbl* were used. These included $trbl^{3\text{-}54}$ containing a P[GawB] P-element insertion ~250 bp 5′ of exon 1 of the *trbl* gene, and two additional alleles $trbl^{1119}$ and $trbl^{3519}$ (Mata et al., 2000; Rorth et al., 2000), which each contain an EP P-element. The $trbl^{1119}$ insertion is ~185 bp downstream of the transcription start site and $trbl^{3519}$ is ~8 bp upstream of the transcription start site. Few progeny can be obtained that are homozygous for either

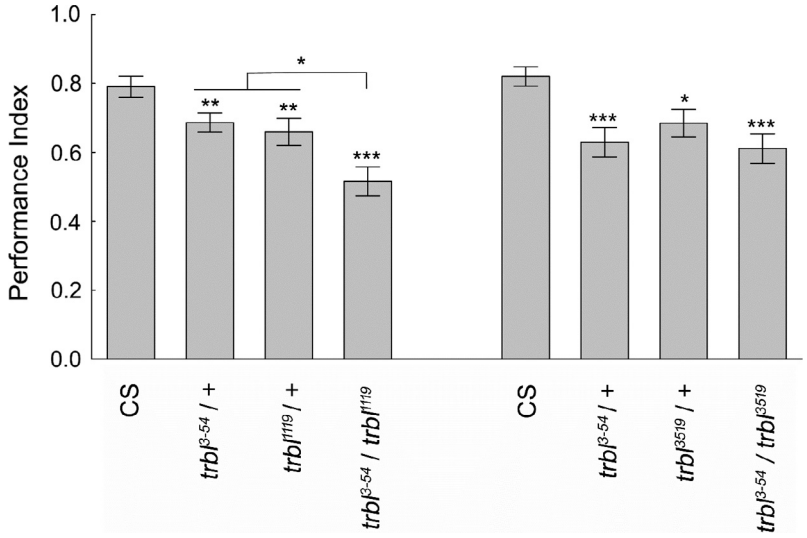

Fig. 1. Complementation test of place memory performance for the *trbl* gene. The memory performance level of each of the fly lines carrying a mutant *trbl* allele was significantly reduced compared to wild type CS. Flies that were trans-heterozygous for the *trbl* gene $trbl^{3\text{-}54}/trbl^{1119}$ performed significantly lower than CS and flies with only one copy of a mutant allele (left-most comparisons $H(3, N = 910) = 53.32$, $p < 0.001$, Kruskal-Wallis ANOVA test with multiple comparisons ($p < 0.05$ = *, $p < 0.01$ = **, and $p < 0.001$ = ***)). The reduction in memory performance found with flies carrying only a single mutant *trbl* allele was not increased with the trans-heterozygote $trbl^{3\text{-}54}/trbl^{3519}$ (right-most comparisons $H(3,N = 750) = 32.67$, $p < 0.001$ Kruskal-Wallis ANOVA test with multiple comparisons ($p < 0.01$ = ** and $p < 0.001$ = ***)). The values represent means and the error bars are SEMs.

trbl^{1119} and *trbl*^{3519}, so all tests performed were trans-heterozygous with the *trbl*$^{3-54}$ allele or with a wild type allele.

3.1. Complementation tests using multiple trbl alleles indicate that the trbl gene is necessary for operant place memory

trbl$^{3-54}$ homozygous flies perform significantly worse than wild-type flies in the operant place conditioning paradigm (LaFerriere et al., 2008). Tests were performed in the heat-box to determine whether two additional alleles might fail to complement the *trbl*$^{3-54}$ phenotype. Following twenty minutes of conditioning using a 41 °C punishment temperature, place memory levels were reduced in all flies that have at least one copy of any of the mutant *trbl* alleles compared to wild type CS flies (Fig. 1). Flies with only one mutant allele of the *trbl* gene had reductions of 13–25% compared to wild-type CS flies' performance. Flies that were trans-heterozygous for *trbl*^{3519}/*trbl*$^{3-54}$ did not have a significant decrease in their performance relative to those carrying only one mutant allele. In contrast, flies of the genotype *trbl*^{1119}/*trbl*$^{3-54}$ performed significantly lower than wild-type as well as the flies heterozygous for other combinations of the mutant *trbl* alleles. The memory of flies of the genotype *trbl*^{1119}/*trbl*$^{3-54}$ relative to wild-type CS was ~30% lower. With the similarity in phenotypes between the flies with the three different insertion alleles of the *trbl* gene and the reduction in performance of one trans-heterozygous combination, these results argue that the *trbl* gene is necessary for operant place memory in the heat-box.

Flies of each genotype were tested for the ability to sense and avoid the high temperatures used in the heat box in a thermosensitivity assay. Flies were allowed to choose between chamber halves that were set at either 24 °C or 41 °C. The only significant difference found was between wild-type CS flies and those trans-heterozygous for *trbl*^{3519}/*trbl*$^{3-54}$ (Table 1). Thus, for the majority of the allelic combinations that were tested there was no significant change in the ability of the flies to sense and avoid the high temperature. There was only one case where a change in the ability to sense temperature could influence place memory. Overall, this indicates that the reductions in memory performance levels with mutation of the *trbl* gene is largely independent from an inability to sense and avoid the high temperature.

3.2. Complementation tests using multiple trbl alleles indicates that the trbl gene is important for aversive olfactory memory

The role of *trbl* in olfactory learning was examined by testing flies using three different mutant *trbl* alleles. *trbl*$^{3-54}$ homozygous flies perform significantly higher than wild-type flies in this paradigm (LaFerriere et al., 2008). Only flies that were trans-heterozygous with two mutant *trbl* alleles performed significantly higher than wild-type CS flies, at ~125% of wild-type levels (Fig. 2).

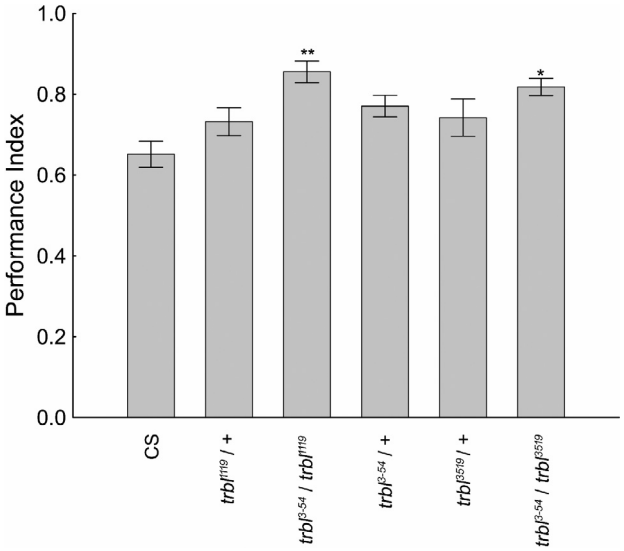

Fig. 2. Complementation test in the olfactory classical conditioning paradigm for the *trbl* gene. Three *trbl* insertion alleles were tested. Complementation tests reveal significantly better memory compared to wild-type CS in the olfactory paradigm for the trans-heterozygotes, *trb*^{1119}/*trbl*$^{3-54}$ and *trbl*^{3519}/*trbl*$^{3-54}$. Flies with a single mutant copy of the *trbl* have increase performance compared to wild-type, but this does not reach significant levels ANOVA: $F(5,58) = 5.04$, $p < 0.001$ Newman-Keuls post-hoc test ($p < 0.05$ = * and $p < 0.01$ = **) N = 8 for each genotype. The values represent means and error bars are SEMs.

Flies with only a single mutant *trbl* allele performed slightly better than wild-type, but never reached statistically significant levels. Thus the *trbl* gene is necessary for the altered phenotypes in olfactory memory tests.

Flies from each genotype were next tested for their ability to sense and avoid the odors and electric shock used for the olfactory aversive conditioning experiment. Although there was variation in the odor avoidance and shock avoidance scores between genotypes, there was only one significant difference found between *trbl*$^{3-54}$/*trbl*^{1119} and CS flies in octanol avoidance (Table 1). A significant difference was not found, however, between flies of other mutant alleles and wild-type, suggesting that there is not a consistent relationship between altered octanol avoidance and olfactory memory in mutant flies. Moreover, changes in olfactory memory levels was not a consequence of changes in the ability to sense and avoid MCH or the shocks used for conditioning.

3.3. The trbl protein is expressed throughout the adult fly brain

The expression pattern of the Trbl protein was examined using a polyclonal antiserum. Anti-Trbl was used to visualize expression

Table 1
Control behaviors in wild-type CS and *trbl* mutant flies.

Genotype	Shock avoidance N = 48	MCH acuity N = 50	Oct acuity N = 54	41 °C avoidance N = 799
CS	73.8 ± 3.1	25.7 ± 4.5	29.4 ± 5.4	0.64 ± 0.03
trbl^{1119}/+	73.8 ± 6.7	15.9 ± 4.2	23.5 ± 5.6	0.64 ± 0.02
trbl$^{3-54}$/*trbl*^{1119}	68.9 ± 7.3	15.0 ± 4.5	8.7 ± 3.9*	0.58 ± 0.02
trbl$^{3-54}$/+	77.2 ± 4.8	12.3 ± 6.8	17.5 ± 4.5	0.63 ± 0.02
trbl^{3519}/+	77.6 ± 3.3	29.4 ± 3.1	10.4 ± 5.5	0.58 ± 0.02
trbl$^{3-54}$/*trbl*^{3519}	77.6 ± 4.2	16.3 ± 3.0	12.5 ± 7.1	0.54 ± 0.02**

Few significant differences were found in the control behaviors of wild-type and *trbl* mutant flies. Shock avoidance: ANOVA $F(5, 42) = 0.40$, $p = 0.85$; MCH acuity: $F(5, 44) = 1.90$ $p = 0.11$; Oct acuity: $F(5, 48) = 2.37$, $p = 0.05$, *trbl*$^{3-54}$/*trbl*^{1119} was significantly different from CS after Multiple Comparisons.

* $p < 0.05$; 41 °C avoidance: Kruskal-Wallis Test $H(5,N = 799)=18.47$, $p = 0.002$, the only significant difference between genotypes after Multiple Comparisons was between CS and *trbl*$^{3-54}$/*trbl*^{3519}.

** $p < 0.01$. The values represent means ± the SEMs.

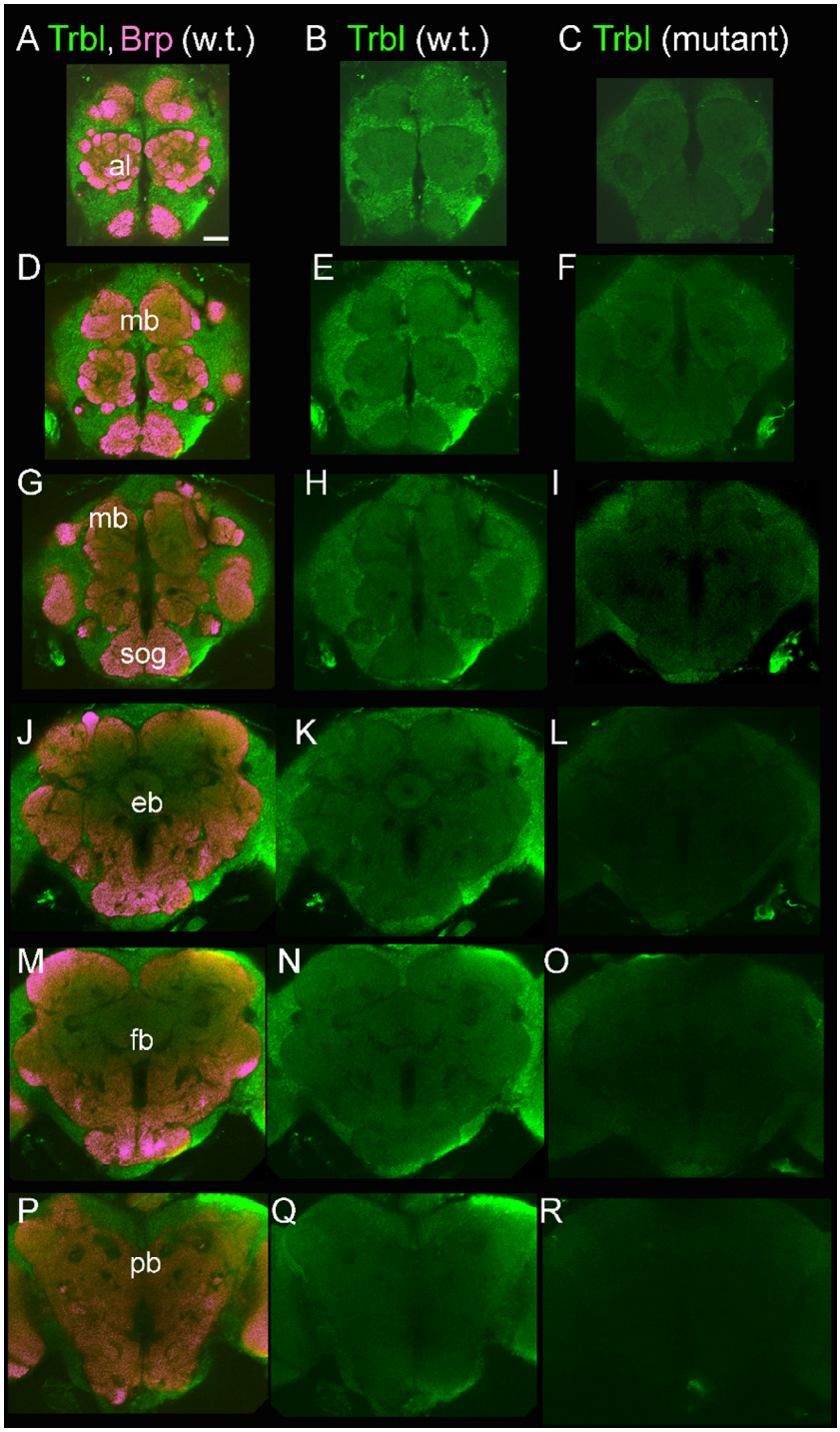

Fig. 3. Tribbles immunostaining in the *Drosophila* brain. Trbl is expressed in cell bodies and the neuropil throughout the fly brain visualized in green in both wild-type CS brains (left and center columns) and *trbl* mutant fly brains (right column). Mutant flies have reduced Tribbles expression compared to wild-type CS levels, comparing center and right most panels. Brains were co-labeled with the *bruchpilot* (*brp*) monoclonal antibody marking the synapses in purple on the left most panels. Labeled structures: antennal lobes (al), mushroom bodies (mb), subesophogial ganglia (sog), ellipsoid body (eb), fan shaped body (fb), protocerebral bridge (pb). Scale bar = 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of Trbl in the adult fly brain of both wild-type CS flies and mutant *trbl* flies (Df(3L)*ri*-79c/*trbl*^{1119}) (Fig. 3). In the CS fly brain, Trbl expression is visible in cell bodies throughout the brain. There is also detectable expression in the neuropil. There is a marked decrease in Trbl expression levels in *trbl* mutant flies. This reduction in anti-Trbl signal between CS flies and *trbl* mutant flies indicates that it is indeed the Trbl protein that is visualized.

3.4. The trbl mutant phenotype can be rescued in operant place memory

The reduced memory phenotype of *trbl* mutant flies in the place-conditioning paradigm was rescued with expression of a wild-type version of *trbl* as a transgene. Since the trans-heterozygote *trbl*^{1119}/*trbl*$^{3\text{-}54}$ gave the strongest mutant phenotype,

these flies along with the appropriate genetic controls were used in the rest of the conditioning experiments. *trbl*$^{3\text{-}54}$ Gal4 driven expression of UAS-*trbl* (Mata et al., 2000) is sufficient to rescue the reduced memory phenotype of *trbl* mutant flies in the heat-box (Fig. 4). We further tested whether the addition of a serotonin Gal4 driver, Trh-Gal4 (Park et al., 2006), could further increase memory levels, since serotonin is the key biogenic amine for operant place memory (Sitaraman et al., 2008). The addition of the Trh-Gal4 driver does not significantly increase the performance of *trbl*$^{3\text{-}54}$-rescued flies (Fig. 4). These results indicate that *trbl* expression is sufficient in cells expressing the *trbl*$^{3\text{-}54}$ Gal4 for normal memory formation.

Flies of the same genotypes used in the rescue experiments were tested for their ability to sense and avoid the high temperature in a thermosensitivity assay. There are no significant differences in thermosensitivity between Trh-Gal4/UAS-*trbl;trbl*^{1119}/*trbl*$^{3\text{-}54}$ and Trh-Gal4/+;*trbl*^{1119}/*trbl*$^{3\text{-}54}$ or between Trh-Gal4/UAS-*trbl;trbl*^{1119}/*trbl*$^{3\text{-}54}$ and UAS-*trbl*/+;*trbl*^{1119}/*trbl*$^{3\text{-}54}$ (Table 2). A significant difference was found between UAS-*trbl*/+;*trbl*^{1119}/*trbl*$^{3\text{-}54}$ and Trh-Gal4/+;*trbl*^{1119}/*trbl*$^{3\text{-}54}$. The flies from one of the genotypes that were rescued for place memory was different from control flies in the ability to avoid high temperatures, but the other rescuing line was not different from the genotypic control for this behavior. These mixed results show that the memory rescue can be dissociated from changes in high temperature avoidance behavior.

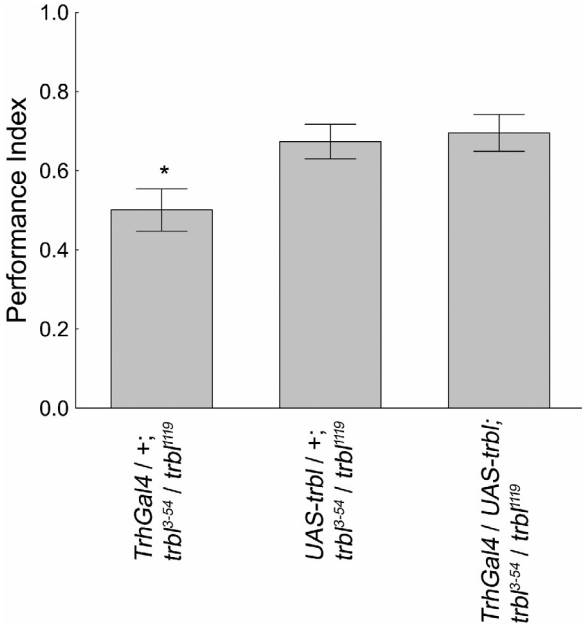

Fig. 4. Rescue of the *trbl* mutant phenotype in the operant place-learning paradigm. *trbl*$^{3\text{-}54}$ driven expression of UAS-*trbl,* in otherwise mutant flies, was able to rescue the *trbl* mutant phenotype in the heat-box. The addition of Trh Gal4 did not increase performance further H(2,N = 476) = 16.18, p < 0.001 Kruskal-Wallis ANOVA. (p < 0.05 = *). The values represent means and error bars are SEMs.

Table 2
Control behaviors for *trbl* mutant rescue in the heat box paradigm.

Genotype	41 °C avoidance N = 357
TrhGal4/+; *trbl*$^{3\text{-}54}$/*trb*^{l1119}	0.67 ± 0.03
UAS-*trbl*/+; *trbl*$^{3\text{-}54}$/*trb*^{l1119}	0.76 ± 0.03
TrhGal4/UAS *trbl*; *trbl*$^{3\text{-}54}$/*trb*^{l1119}	0.71 ± 0.03

Kruskal-Wallis Test; H(2,N = 357) = 15.43 p = 0.0004. A significant difference was found between UAS-*trbl*/+; *trbl*$^{3\text{-}54}$/*trb*^{l1119} and *TrhGal4*/+; *trbl*$^{3\text{-}54}$/*trb*^{l1119} p < 0.001 after Multiple Comparisons for 41 °C avoidance. No other significant differences were found.

3.5. The expression pattern of the trbl$^{3\text{-}54}$ Gal4 driver examined with UAS-GFP

The expression pattern of the *trbl*$^{3\text{-}54}$ Gal4 driver was examined using UAS-GFP driven expression. This was done to find where *trbl* expression is sufficient to rescue the reduced memory phenotype in the heat-box. Two different UAS-GFP fly lines were used, UAS-nls-GFP (targeting GFP expression to the nucleus) and UAS-mCD8-GFP (targeting GFP expression to the membranes) (Lee & Luo, 1999; Neufeld, de la Cruz, Johnston, & Edgar, 1998). Expression is visualized throughout the brain (Fig. 5). Expression in the antennal lobes and the antennal nerve are visible in the anterior most sections using either UAS-GFP (Fig. 5). More posterior, expression in the median bundle and the ellipsoid body is visible along with a cluster of cells on the dorsal surface of the brain in the pars intercerebralis (Fig. 5). The pars intercerebralis cluster of cell bodies becomes more noticeable moving farther posterior to the level of the fan shaped body (Fig. 5). The most posterior sections have several cell bodies present (Fig. 5). There is also expression around the periphery of the whole brain in all sections with UAS-mCD8-GFP, which may correspond to glial cells. Although unlikely, we cannot rule out the possibility that expression outside of the nervous system could be important for the *trbl*-dependent function in behavior.

3.6. The trbl mutant phenotype can be rescued for aversive olfactory memory

Experiments to attempt a genetic rescue of the increased memory phenotype of *trbl* mutant flies in olfactory aversive conditioning paradigm were performed. The trans-heterozygous *trbl*^{1119}/*trbl*$^{3\text{-}54}$ flies gave the strongest mutant phenotype, so they were used as the *trbl* mutant flies in the rest of the olfactory conditioning experiments. The *trbl*$^{3\text{-}54}$ driven expression of UAS-*trbl* was not sufficient to rescue the olfactory memory changes (Fig. 6). Addition of the *c155*Gal4 driver, by contrast, did rescue the elevated olfactory memory scores of *trbl* mutant flies.

The same genotypes of flies used for the olfactory aversive rescue experiments were examined for their ability to sense and avoid the odors and electric shock used during the experiment (Table 3). The only significant difference found was between *c155Gal4/+*; UAS-*trbl*/+;*trbl*^{1119}/*trbl*$^{3\text{-}54}$ and UAS-*trbl*/+;*trbl*^{1119}/*trbl*$^{3\text{-}54}$ for MCH avoidance. This difference is unlikely to cause the memory phenotypes observed, since this difference in MCH acuity does not seem to be related with the altered memory performance index.

4. Discussion

Although the *trbl* pseudokinase in *Drosophila*, and the vertebrate *trbl* 1–3, are expressed in the brain (Fig. 3) (Aime et al., 2015; Fisher et al., 2012; Ord et al., 2014), the results here provide the first definitive evidence for the function of this protein in regulating behavior. We show that the *trbl* gene is critical for normal memory formation. That is, the *trbl* gene provides a function that permits memories to be formed normally. The results here cannot discriminate a function for *trbl* in different components or stages of memory formation. Mutation of the *trbl* gene causes opposite effects in the two conditioning paradigms tested. Nevertheless, when multiple alleles are tested each results in abnormal phenotypes in the two paradigms. A decrease in memory performance in operant place conditioning was identified in flies heterozygous for three different *trbl* alleles. In one case, this defect was increased in flies

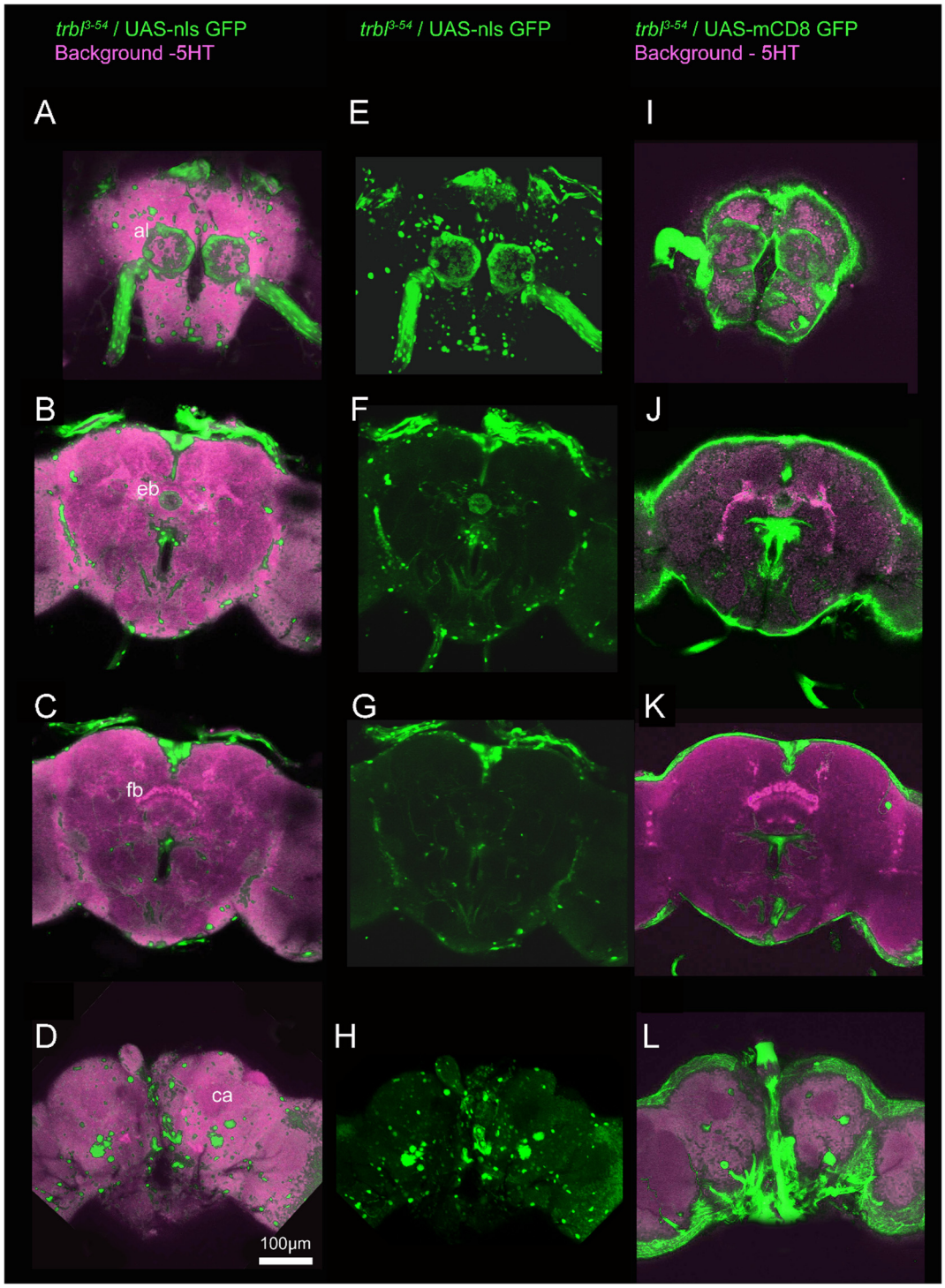

Fig. 5. *trbl*$^{3\text{-}54}$ Gal4 driven expression of two different UAS-GFPs in the adult brain. GFP expression is visualized from anterior to posterior A-D with UAS-nls-GFP and anti-5HT, E-H with UAS-nls-GFP alone, and I-L with UAS-mCD8-GFP and anti-5HT. Notable structures are labeled: antennal lobes (al), median bundle (meb), ellipsoid body (eb), fan shaped body (fb) and calyces (ca). Expression is visualized throughout the brain including in the antennal lobes, median bundle, ellipsoid body, and around the periphery of the brain. Scale bar = 100 µm.

trans-heterozygous for two different mutant *trbl* alleles. Conversely, an increase in memory performance in classical olfactory aversive conditioning was observed only for flies that were trans-heterozygous for two different mutant alleles. The memory phenotypes from flies with multiple mutant alleles argues that the *trbl* gene is necessary for normal memory formation. Moreover, the *trbl* mutant phenotype is rescued by targeted gene expression in both of the conditioning paradigms tested. Using transgenic expression of a UAS-*trbl* gene, we are able to rescue place memory using *trbl*$^{3\text{-}54}$ Gal4 driven expression. The addition of a Trh-Gal4 did not increase the memory performance further relative to expression driven by *trbl*$^{3\text{-}54}$ Gal4 alone. We were also able to rescue aversive olfactory memory using *c155*Gal4 driven expression. *c155*Gal4 is expressed throughout the nervous system (Lin & Goodman, 1994). Since expression of UAS-*trbl* in neurons is sufficient to normalize aversive olfactory memory, we conclude that *trbl* expres-

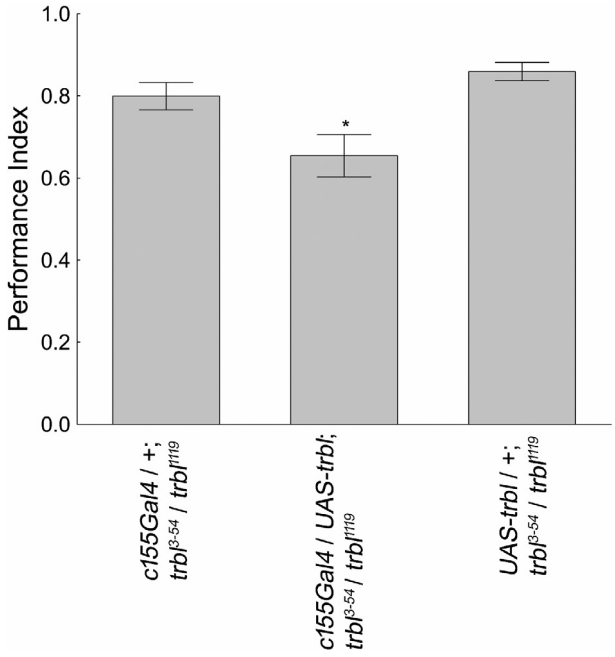

Fig. 6. Rescue of the *trbl* mutant phenotype in olfactory aversive conditioning. *c155*Gal4 driven expression of UAS-*trbl*, in otherwise mutant flies was able to rescue the *trbl* mutant phenotype in the olfactory conditioning paradigm, causing these flies to have significantly lower memory scores compared to the control genotypes. ANOVA: $F(2,18) = 7.83$, $p = 0.004$ Newman-Keuls post-hoc test ($p < 0.05 = *$). N = 7 for each group. The values represent means and error bars are SEMs.

Table 3
Control behaviors for *trbl* mutant rescue in the aversive olfactory paradigm.

Genotype	Shock acuity N = 18	MCH acuity N = 18	Oct acuity N = 12
c155Gal4/+; trbl$^{3-54}$/trbl^{1119}	72.6 ± 5.8	−4.4 ± 2.2	11.1 ± 9.2
UAS-*trbl/+; trbl$^{3-54}$/trbl^{1119}*	65.4 ± 5.7	9.6 ± 4.3	24.7 ± 8.3
*c155Gal4/+;*UAS-*trbl/+; trbl$^{3-54}$/trbl^{1119}*	52.4 ± 7.4	−4.1 ± 6.7	12.8 ± 13.2

Only a single significant difference was found for the aversive olfactory rescue experiment control behaviors. Shock avoidance: ANOVA $F(2,15) = 2.6$, $p = 0.11$; MCH acuity: $F(2,12) = 4.4$, $p = 0.038$; Oct acuity: $F(2,9) = 0.5$, $p = 0.62$. MCH acuity is significantly different between *c155Gal4/+;*UAS-*trbl/+; trbl$^{3-54}$/trbl^{1119}* and UAS-*trbl/+; trbl$^{3-54}$/trbl^{1119}*, $p = 0.04$ following Newman-Keuls *post-hoc* test. The values represent means ± the SEMs.

sion in neurons of the brain is important for aversive olfactory memory formation. Thus, using multiple allelic combinations and transgenic rescue, the *Drosophila trbl* gene is clearly important for memory formation.

That *trbl* mutation causes opposite phenotypes when tested in these two paradigms is rare but not unique. Behavioral changes such as these have been observed in flies mutant for the *white* ABC transporter (Diegelmann et al., 2006). The mutant *white* gene causes defects in both place memory and aversive olfactory memory. For place memory, flies' mutant for *white* perform significantly worse than wild type, but when tested using different shock intensities for olfactory aversive memory, mutant flies perform significantly better than wild type. Both serotonin and dopamine levels are reduced by mutation of the *white* ABC transporter and these biogenic amines play a role in place and olfactory memory (Sitaraman et al., 2008; Waddell, 2010). Perhaps the *trbl* gene alters the biogenic amine systems or its effects to alter memory formation. Future experiments testing the role *trbl* in biogenic amine systems will address this possibility.

There are two primary open questions about the function of *trbl* in memory formation. Where in the fly brain *trbl* is acting to regulate memory formation is not clear. The expression pattern of Trbl is throughout the adult brain (Fig. 3). The Trbl signal is strongest in the cell body rind. Weaker but clear expression is detected throughout the neuropil. There was no region with relatively high or low expression that would provide a clue to where Trbl might be acting in memory formation. At a minimum one can conclude that Trbl is expressed in brain regions that have been previously shown as important for both place and olfactory memory, including the mushroom bodies and the median bundle (Guven-Ozkan & Davis, 2014; Heisenberg, 2003; Zars, Wolf, et al., 2000; Zars, Fischer, et al., 2000; Zars, 2000). The rescue of the place memory phenotype with *trbl$^{3-54}$* suggests regions that might be important for this type of conditioning. Gene expression driven by *trbl$^{3-54}$* GAL4 was detected in the antennal lobe, ellipsoid body, and median bundle (Fig. 5). These are the same regions that have been shown to be important for *rutabaga*-dependent place memory (Zars, Wolf, et al., 2000). That the olfactory memory phenotype could only be rescued with the addition wide-spread expression in the nervous system suggests that *trbl* expression is required in places in addition to the antennal lobe, ellipsoid body, and median bundle. Or, alternatively, that there is more expression of UAS-*trbl* in the *trbl$^{3-54}$* GAL4 positive cells with the addition of the *c155*GAL4 driver. In any event, a good candidate structure for local rescue of *trbl* would be in the mushroom bodies, as these have been repeatedly shown as a site critical for olfactory memory (Guven-Ozkan & Davis, 2014; Heisenberg, 2003; Zars, Fischer, et al., 2000; Zars et al., 2000). When in the life cycle *trbl* is acting to support normal memory is also not clear. The *trbl* gene product has multiple cellular functions and a role in early development (Eyers et al., 2017; Masoner et al., 2013). One cannot differentiate with the current results whether or not *trbl* acts through development or in the adult fly for memory formation. That there is widespread expression of the Trbl protein in the fly brain suggests that there will be adult-specific functions of this protein. Future investigation of *trbl* will be needed to address where and when this gene is sufficient for proper memory formation.

The finding that *trbl* is critical for place memory and olfactory memory suggests a new set of signaling mechanisms that could be important for memory formation. The *trbl* gene encodes a pseudokinase. The conserved serine/threonine kinase domain is missing key residues necessary for normal kinase activity including an ATP binding region. Observed interactions indicate that *trbl* acts as a signal transducer to target specific proteins to a ubiquitin ligase followed by protein degradation (Eyers et al., 2017; Mata et al., 2000; Qi et al., 2006). In *Drosophila, trbl* is a known regulator of both *string/CDC25* (a cell cycle regulator) and *slow boarders* (a homolog to the C/EBP family of transcription factors) by targeting each to the proteasome pathway (Masoner et al., 2013; Mata et al., 2000; Rorth et al., 2000). In mammals, *trbl* homologous gene products regulate the activity of a number of proteins. Mammalian Trbl proteins are implicated in regulating AKT/FOXO and MAPK signaling in various cancer models (Du, Herzig, Kulkarni, & Montminy, 2003; Erazo et al., 2016; Salazar et al., 2015; Sung et al., 2007; Yokoyama et al., 2010; Zareen, Biswas, & Greene, 2013). They also play a role in regulation of transcription factors like ATF4 and C/EBPalpha (Bowers, Scully, & Boylan, 2003; Dedhia et al., 2010) and interact with a number of additional proteins including p65 (regulating NF-kappaB-dependent transcription), CtIP (cell cycle regulation), and MAPKK (Hegedus, Czibula, & Kiss-Toth, 2007; Kiss-Toth et al., 2004; Wu, Xu, Zhai, & Shu, 2003; Xu et al., 2007). The *trbl*-dependent signaling functions from other systems provide a rich target for novel memory formation mechanisms in *Drosophila* and across species.

To summarize, flies mutant for multiple *trbl* alleles show abnormalities in two forms of memory. Transgenic rescue with a wild-type version of the *trbl* gene reverses the mutant phenotype to

wild-type levels. Expression analysis shows widespread expression of Trbl in the fly brain. Thus, the *trbl* pseudokinase can be critical for regulating complex behavior.

Funding resources

Research in the laboratory of TZ is funded by the National Science Foundation [Grants 1535790 and 1654866] and National Institutes of Health [Grants DK107900 and NS076980].

Acknowledgements

The authors thank Pernille Rorth and Leonard Dobens for kindly providing fly strains. The Bloomington *Drosophila* Stock also provided fly strains.

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