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

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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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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.