The Drosophila Gene RanBPM Functions in the Mushroom Body to Regulate Larval Behavior

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Abstract

Background: In vertebrates, Ran-Binding Protein in the Microtubule Organizing Center (RanBPM) appears to function as a scaffolding protein in a variety of signal transduction pathways. In Drosophila, RanBPM is implicated in the regulation of germ line stem cell (GSC) niche organization in the ovary. Here, we address the role of RanBPM in nervous system function in the context of Drosophila larval behavior.

Methodology/Principal Findings: We report that in Drosophila, RanBPM is required for larval feeding, light-induced changes in locomotion, and viability. RanBPM is highly expressed in the Kenyon cells of the larval mushroom body (MB), a structure well studied for its role in associative learning in Drosophila and other insects. RanBPM mutants do not display major disruption in nervous system morphology besides reduced proliferation. Expression of the RanBPM gene in the Kenyon cells is sufficient to rescue all behavioral phenotypes. Through genetic epistasis experiments, we demonstrate that RanBPM participates with the Drosophila orthologue of the Fragile X Mental Retardation Protein (FMRP) in the development of neuromuscular junction (NMJ).

Conclusions/Significance: We demonstrate that the RanBPM gene functions in the MB neurons for larval behavior. Our results suggest a role for this gene in an FMRP-dependent process. Taken together our findings point to a novel role for the MB in larval behavior.

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Introduction

The fruitfly Drosophila melanogaster has been employed successfully as a genetically tractable model for the study of nervous system development and function. A large fraction of behavioral studies in Drosophila have focused on the adult fly. However, the relative complexity of the adult nervous system makes the identification of neural networks involved in the behavioral challenges. More recently, the Drosophila larva has emerged as a simpler model for the identification of the neural circuitry underlying behaviors such as learning and memory [1,2] and feeding [3,4].

The Drosophila foraging larva lives in the food source. It feeds constantly until it wanders off the food substrate, in search for a site to undergo metamorphosis. Consistently, throughout the foraging stage of larval development Drosophila is repelled by light and continuously attracted to food. As the larva enters the wandering stage food intake ceases, the gut is emptied and it becomes averse to food and indifferent to light [5,6].

We took a genetic approach toward the identification of neurons that play a role in the control of locomotion. Control of locomotion was studied in the context of light induced changes to larval movement that reflect the Drosophila larva’s repulsion from light. This approach led to the identification of a transposable element mutation that caused severe disruption in larval response to light as measured in this assay as well as in locomotion, feeding behavior, and lethality. The P-element insertion disrupts the Drosophila orthologue of the vertebrate Ran-Binding Protein in the Microtubule Organizing Center (RanBPM) gene, originally identified in a yeast two-hybrid screen using Ran GTPase as bait [7]. Function of RanBPM in Ran-dependent processes and in the centrosome has not been established. In Drosophila, RanBPM is required in the ovary for the regulation of germ line stem cell (GSC) niche organization [8].

RanBPM protein contains multiple conserved domains implicated in protein-protein interactions as a SPRY domain found Sp1A and ryanodine receptors, a lysenchephaly homology...
(LisH) motif, a motif C-terminal to LisH (CTH), and a CRA (CT11-RanBPM) domain. RanBPM binds to and regulates the function of a variety of proteins e.g. [9,10,11,12,13,14,15]. The functional relevance of several of these interactions is yet to be established. Of interest is the reported RanBPM-mediated regulation of TrkA and PlexinA receptors [16,17]. Collectively these observations suggest a role for RanBPM as a scaffolding protein.

One potential partner of RanBPM, as established by in vitro binding assays, is the Fragile X Mental Retardation Protein (FMRP) [18]. Lack of FMRP function is the underlying cause of the more prevalent form of inherited mental retardation [19]. FMRP is an RNA binding protein, highly conserved and required for mRNA transport and translational suppression in the context of synaptic plasticity. In Drosophila as well as other model systems, lack of the Drosophila orthologue gene (dfmr1) causes excess synaptic elaboration [20,21,22] and disruption in activity-dependent plasticity [23,24].

Using a number of different genetic and cell biology tools we demonstrate that in Drosophila RanBPM function is required in the nervous system for the modulation of locomotion by light, feeding behavior and growth. RanBPM is highly expressed in the Kenyon cells of the mushroom bodies (MB) and targeted expression in these neurons is sufficient to rescue all behavioral phenotypes of the mutant larvae. RanBPM mutations do not appear to disrupt basic aspects of nervous system development.

The MB is a prominent neuropil structure implicated in olfactory learning and memory, as well as other complex behaviors of the adult fly (e.g. reviewed by [25,26]). MB function in larval behavior has not been as extensively studied. Recent investigations indicate a role for MB output in a model for associative learning in the third instar larva e.g. [1,27].

As a first step toward the identification of the signal transduction pathways supported by RanBPM function, we sought evidence for an interaction with proteins previously identified, in vertebrates, as potential partners of RanBPM. We found that reduction of RanBPM function suppresses the neuromuscular junction (NMJ) overgrowth phenotype caused by mutations in the Drosophila orthologue of FMRP (dfmr1) suggesting that RanBPM protein may contribute to FMRP-dependent processes. Taken together our results demonstrate that RanBPM function in the MB contributes to the regulation of larval behavior and suggest a novel role for this structure.

Materials and Methods

**Drosophila strains and culture**

Synchronized larvae at the early third instar foraging stage were obtained as described [6] and grown at 25 °C unless otherwise stated, in light/dark cycles in food supplemented with vitamin A (1.25 g/L). Mutations were kept over a CrI(Y) balancer in a yw background for the identification of homozygous mutants by the ON/OFF assay [28]. RanBPM s135 is a deletion that eliminates a maximum of 4.1 kb from the N-terminus to the middle of the gene (6474 to 68882 in AE003829), generated by excision of the ON/OFF construct was provided by Paul Lasko (McGill University, Canada). The UAS-RanBPM/ls line was generated from the cDNA clone RH161511 (FB30729865, http://flybase.org/). All constructs were sequenced. fmr1Ep517 (BSC, stock #6928), is a hypomorphic allele, [32] and fmr1A5564 (BSC, stock #6930) is a deletion [32].

**Behavioral Assays**

**ON/OFF.** We used the ON/OFF assay to measure changes in locomotion in response to light in individual larvae as described [33,34]. Larvae were manipulated in darkness except for a red safelight (20 W lamp with GX-2 filter KODAK). For the assay, a single foraging third instar larva (84–90 h AEL) was placed in the center of the plate and subjected to 10 sec of light and 10 sec of dark and so forth. The light, cool white bulbs, (15 W, Sylvania in a Rapid Start mechanism), was controlled by a serial microcontroller (MacIo, MacBrick) and a relay unit (AZ696) connected to a computer that ran a custom macro (NIH Image 1.62f). A Fujinon TV-Z zoom lens attached to a CCD TV camera (Elmo, TSE 2728) captured behavior. Each trial lasted 60 or 120 seconds. The semi-automated system described in detail elsewhere [6,33,35] was used to track and calculate the Response Index (RI) (RI = [path length in dark – path length in light]/total path length during the assay]) through the execution of a NIH Image macro. Alternatively, larval movement during the assay was analysed using Dynamic Image Analysis System (DILAS) software [36,37]. The parameters and methodology used as previously described [34]. Briefly, centroid position over time was used to generate a larval path employed to calculate change of direction. For a qualitative analysis, perimeter stacks (larval outlines) of representative larvae were generated. All genotypes were tested for locomotion in constant dark (safe-light) as described above using the semi-automated system.

**Feeding assays.** The food intake assay was conducted as in [38] with batches of 25 larvae staged as described. Larvae were starved for two hours and placed in a drop of yeast paste laced with blue food dye for 30 min or 1.5 hours (Food Dispersal). At that the end of the assay the fraction of larvae displaying blue gut and the fraction immersed in the yeast were determined. Alternatively, larvae were placed 3.99 cm (+/-0.04, N = 7) away from the centrally-located yeast drop (Food Attraction). After 1.5 hours the fraction displaying blue gut and the fraction immersed in the yeast were determined as above. Larval behavior was captured (6 frames/min) for the duration of the assay.

**Contact chemosensory assay.** Response to NaCl was assayed essentially as described in [39] and [40]. Approximately 25 larvae were placed in the center of a petri dish divided into 4 quadrants. Opposite quadrants were filled with 1% agar in 1 M NaCl or 1% agar in water. The position of each larva was determined at 10 and 15 min intervals. Larvae that did not move outside a 1 cm radius from the original position were not counted. Similar fraction of yw, Revertants, RanBPM mutant and larvae remained inside the 1 cm (30–20%), while OR mostly moved out of this area by 10 min (93%).

**Statistical Analyses**

We used SPSS Version 17 and The SAS System Version 9.2. The statistical tests included one-way analysis of variance (ANOVA) and Tukey’s-pairwise comparisons. In SAS we used the GENMOD Procedure with a Binomial distribution and a Link
Function called ‘Logit’ to conduct a form of chi square analysis for nonparametric data. Normality tests on the residuals of the ANOVAs were conducted using the Shapiro-Wilk test. Verification of samples was performed by the F-test or Bartlett’s test. The level of significance $x$ in all tests was 0.05. All measurements are shown as mean values and SEM, and * indicates samples significantly different from control genotypes.

**Immunohistochemistry and imaging**

**CNS.** Larval brains from foraging third instar larvae staged as above were treated as described [35]. Primary antibodies used were: mouse anti-Elav (1:200), anti–Repo (1:200) and anti-FasII (1:2) (DSHB), rabbit anti-RanBPM, (1:1000) [8], rabbit anti-β-galactosidase (1:100) (Cappel), rabbit anti-S-HT (1:200) (Protos Biotech), rabbit anti-phosphorylated Histone3 (Upstate Biotechnologies 1:1000) and rabbit anti-Rfamide (1:1000) (SNF, kindly donated by Jan Veenstra). The anti-RanBPM antibody was generated in rabbits against a N-terminal fragment of RanBPM. In western blotting it recognizes in wild type samples a single band of approximately 140 kDa absent in samples prepared from deletion strains [8]). Secondary antibodies used: Texas Red-conjugated goat anti-rabbit IgG (1:200), HRP-conjugated goat anti-mouse IgG and Cy3-conjugated goat anti-rabbit IgG (1:200) (Jackson); Alexa 594-conjugated goat anti-mouse IgG (1:250), Alexa 488-conjugated goat anti-mouse IgG (1:200) (Molecular Probes).

**NMJ.** Foraging third instar larvae were dissected in phosphate buffered saline in order to expose the musculature of the body wall, fixed at room temperature in 4% formaldehyde in PBS and buffered saline in order to expose the musculature of the body wall. Z-stack of the NMJ of muscles 6 and 7 were projected as a single image and the total number of boutons and branches counted.

**Confocal.** We used a Zeiss Axiovert 100 M and the LSM 510 image software. Brightness and contrast were adjusted using Adobe Photoshop 5.0.

**Results**

**Mutations in the RanBPM gene cause disruption of the larval response to light and locomotion**

The third instar larva light avoidance response is characterized by interruption of forward peristalsis, vigorous head-swinging followed by direction change, leading to a reduction in locomotion ([6,34] and Figure 1C). Light avoidance can be measured in the ON/OFF assay, as changes to different aspects of locomotion of a single larva exposed to intermittent pulses of light [35,34].

Larvae homozygous for a P-element insertion in the predicted second exon of the RanBPM gene [8], henceforth named RanBPM[60220] or for alleles generated by imprecise excision of this element, displayed a response index in the ON/OFF assay (RI = [(path length in dark – path length in light)/total path length during the assay]) which was significantly lower than that of controls (Figure 1A). DIAS-assisted analysis of larval locomotion during the assay revealed that in these mutants locomotion is reduced and uncoordinated as seen by the equally high degree of direction change during the lights on and off pulses (Figure 1B, C and D). RanBPM[60220] mutant larvae also displayed reduced locomotion, in constant dark (Figure S1).

The RanBPM gene was previously characterized as a vital gene (Table S1) playing a role in ovary development [8]. A deletion of the upstream region, (RanBPM–) generated by these authors was used in behavioral assays as described above. Our results suggest that RanBPM– allele represents a less severe disruption of the RanBPM gene function. While larvae homozygous for the RanBPM[60220] allele show a reduction in the response to light similar to that exhibited by larvae carrying the RanBPM[60220] allele (Figure 1A), these mutants move more vigorously than the others (143.88 pixels +/-12.41 versus 264 +/-3.96 for RanBPM[60220] N>15). We conclude that RanBPM gene function is required for larval response to light and for coordinated locomotion.

**RanBPM function is required for larval feeding behavior**

Larvae homozygous for either the RanBPM[60220] or RanBPM[60220] alleles, but not for the RanBPM[60220] allele, were smaller than yw control specimens at chronologically the same stage (Figure S2 and data not shown) This size difference was apparent from the early third instar stage but not before.

Reduced larval growth can occur as a consequence of decreased food intake, arrest of cellular growth or a decrease in endoreplication [3,41,42,43,44]. In order to address whether insufficient food intake is associated with the reduced size of RanBPM mutants, we conducted feeding assays essentially as described previously [38].

Following a 2-hour starvation period, early foraging third instar RanBPM mutants were placed on yeast paste containing food dye for 30 min. While circa 88% of the control larvae ingested food within 30 minutes of being placed on the yeast paste, only 14–30% of RanBPM mutants (RanBPM[60220] and RanBPM[60220] homozygous or heteroallelic combinations) displayed the characteristic blue gut (Table 1 and Table S2). As expected, given its normal size, mutant larval homozygous for the RanBPM[60220] allele ate like the control genotypes (Table S2).

To further investigate the feeding phenotype of RanBPM mutants, we allowed larvae to feed for a longer period (1.5 hours) The fraction remaining in the food and the fraction displaying a blue gut were determined as above. Control larvae, vigorously ingested food and remained for the most part immersed in the yeast for the duration of the assay. In contrast, a large fraction of RanBPM mutant larvae left the yeast drop and were found at the end of the assay outside the food source (Figure 2A).

In order to determine whether the RanBPM mutants were attracted to yeast we placed the larvae outside the centrally located yeast plug. After 1.5 hours the fraction that moved into and remained in the food and the fraction displaying a blue gut was determined as above (Figure 2B). Larval behaviour during the assay was captured and a movie of a representative assay is available as supplementary material (Movies S1 and S2).

As expected, at the end of the assay, a large fraction of the control larvae were found immersed in the food and displayed the characteristic blue gut of fed larvae. In contrast, a large fraction of the RanBPM mutants did not move into the food source or did not eat (Figure 2B). The behavior of RanBPM mutants captured during the assay showed that these larvae moved around the plate and came close to the yeast drop but rarely entered and/or remained immersed in it (Movie S2). Interestingly, lack of RanBPM function does not impair the larva’s repulsion to high concentrations of NaCl (Figure S3).

The apparent lack of interest in food displayed by RanBPM mutant larvae is reminiscent of precocious wandering behavior typical of the older third instar larvae, suggesting that lack of RanBPM function disrupts processes that underlie food intake. The multiple phenotypes displayed by RanBPM mutants suggest that this is a pleiotropic gene required in a variety of tissues. Alternatively, these phenotypes may be due to lack of gene function in a defined group of cells.
RanBPM mutations cause reduction in cell proliferation but no apparent disruption in differentiation of identified larval neurons.

We asked whether reduced larval growth was associated with reduced cell proliferation as seen by phosphorylated Histone3 (phosphoH3) immunolabelling of whole-mount RanBPM mutant larval brains. Indeed, smaller than wild-type brains of larvae homozygous for RanBPMK05201 showed a dramatic reduction in phosphoH3 immunolabelling (Figure 3). Surprisingly, we did not detect major morphological defects in these mutant larvae. Labeling of dissected larval brains with a number of different neuronal markers such as 5-HT [45], FasII [46], sNPF [47,48], 386-GAL4 [31], and 247-GAL4 [49,50], NPF [51] indicated that RanBPM is not required for the differentiation and/or maintenance of larval neurons (Figure S4 and data not shown). The smaller size of the CNS is reflected in the apparent smaller volume of the MB neuropil (Figure S4 A, A′). Moreover, we detected a small but statistically significant reduction in the number of serotonergic cell bodies with no apparent disruption in the pattern of projection of these neurons (Figure S4 H–J).

The long isoform of RanBPM is highly expressed in the Kenyon neurons of the mushroom body.

The RanBPM gene encodes two protein isoforms with predicted masses of 67 and 140 kD (named RanBPMshort, RanBPMlong). Both proteins contain the SPRY domain, LisH motif, CTLH motif and the CRA domain. The long isoform differs by the presence on the N terminus of a non-conserved glutamine rich segment [8].

Figure 1. Mutations in RanBPM gene impair larval response to light and locomotion. Response to light is seen in the ON/OFF assay as light-induced changes to quantitative aspects of locomotion. This response was quantitated in the semi-automated system as a response index (RI = [Distance traveled (pixels) in dark (OFF) pulses - distance traveled (pixels) in light (ON)]/Total distance traveled) (A). DIAS was used to calculate direction change (B) or response of an individual larva during the course of the assay (C and D). The latter is shown as empty larval outlines (perimeter stacks) depicting behavior during the light (ON) pulse, and shaded outlines behavior during the dark (OFF) pulse. Quantitative analysis of perimeter stacks are shown in the graphs below as centroid translocation (mm) and direction change (deg). Points below the 20° threshold (dashed line) indicate linear movement. Reduced RanBPM function disrupts the larval response to light as seen by the significant lower RI exhibited by homozygous RanBPMK05201, RanBPMK05201 or S135 (p < 0.05). The response measured for homozygous RanBPMK05201 is markedly reduced but not statistically significant (p = 0.102) nevertheless this allele does not complement the RanBPMK05201 (ANOVA F(7,131) = 7.242, p < 0.0001, A). The control genotypes (OR, yw and RanBPMrevertant) show the increased change of direction triggered by light, that characterizes the photophobic behavior of the foraging larva (B and C). In contrast, in the RanBPMK05201 mutants not only is locomotion markedly reduced but it is also uncoordinated as seen by the significant higher change of direction that occurs during the dark and light pulses (B p < 0.001 and D). (ANOVA for direction change during light F(3,30) = 5.934, p < 0.03 and during dark F(3,30) = 25.351, p < 0.0001, B). In all genotypes N=12. doi:10.1371/journal.pone.0010652.g001
To investigate the expression of the RanBPM gene we used an antibody directed against the N-terminus unique to the 105 kDa isoform kindly provided by Paul Lasko (McGill University,[8]). In the brain lobes and ventral nerve cord (VNC), RanBPM was expressed widely but not in all neurons labeled by the pan-neural marker elav[52,53]. No staining was observed in the proliferating centers of the optic lobe or in the photoreceptors (Figure 4A).

Table 1. Feeding Rescue.

|                  | Yw               | RanBPM<sup>k05201</sup> | RanBPM<sup>k05201</sup>; UAS-RanBPM |
|------------------|------------------|-------------------------|--------------------------------------|
|                  |                  |                         |                                      |
|                  |                  | Long                    | Short                                |
| yw               | 88.667 ± 0.882   | 29.67 ± 4.26            | 25.6 ± 1.89                          |
| RanBPM<sup>k05201</sup> | 33 ± 2.73        | 83 ± 2.65               | 86.33 ± 3.71                         |
| RanBPM<sup>k05201</sup>; elav-GAL4 | 27 ± 3.16        | 64 ± 2.65               | 81.2 ± 2.31                          |
| RanBPM<sup>k05201</sup>; 247-GAL4 | 24.7 ± 4.12      | 35 ± 4.16               | 85 ± 3.06                            |
| RanBPM<sup>k05201</sup>;386-GAL4 | 37 ± 4.09        | 83.33 ± 2.6             | 71.67 ± 1.45                         |

RanBPM<sup>k05201</sup> homozygous mutants had a significant reduction in presence of blue guts compared to wild type YW (χ² (15) = 426.63 p < 0.00001). RanBPM<sup>k05201</sup> homozygous mutants, those carrying a GAL4 driver alone or those carrying UAS-RanBPM short or long alone were not significantly different from one another. elav-GAL4 (p < 0.0001), MB247-Gal4 (p < 0.0001), Dmef2-Gal4 (p = 0.0001), were able to rescue the feeding phenotype with both RanBPM short and long isoforms. 386-GAL4 was able to rescue the feeding phenotype with the short isoform (p < 0.0001) but not the long (p = 0.0719).

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Consistent with this observation, no co-localization was detected.

Figure 2. RanBPM<sup>k05201</sup> mutants feed less and are not attracted to food. Batches of 25 3rd instar foraging larvae were starved for 2 hours and then placed inside (A, Food Dispersal) or outside the food (B, Food Attraction) laced with blue dye for 1.5 hours. The proportion of larvae that were in the food was determined at the end of the assay. All larvae were examined for the presence of blue matter in the gut indicative of food intake. RanBPM<sup>k05201</sup> mutants when placed inside the food in the beginning of the assay ingest food significantly less than control larvae (A shaded bars, χ² = 94.68, DF = 3, p = 0.0001) and are found immersed in the food in a significantly smaller number than control (A empty bars, χ² = 83.31 DF = 3, p = 0.0001). Similarly, when placed outside the food plug RanBPM<sup>k05201</sup> mutants feed significantly less (B shaded bars, χ² = 120.89, DF = 3,) and are attracted to the food less (B empty bars χ² = 121.09 DF = 3) when compared to control larvae. In all experiments * p < 0.0001, N > 100.

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Expression of either RanBPM isoform under the control of the tub-GAL4 driver reduced the recessive lethality associated with RanBPM mutations from 100% to 61–69% (N>200), demonstrating that the both constructs contained all the information required to restore RanBPM gene function and that the two isoforms are functionally equivalent.

Next, we asked whether expression in the nervous system was sufficient to rescue RanBPM phenotypes. Targeted expression of either the short or long RanBPM isoform using the pan-neural driver embryonic lethal abnormal visual system (elav)-GAL4 effectively rescued the response to light phenotype of RanBPM mutants (Figure 5A) as well as the feeding phenotype (Table 1). Using the elav-GAL4 driver overall locomotion of RanBPM mutant larvae markedly improved but was only significantly above background when the long isoform construct was used. (Figure 5B). Interestingly lethality was partially rescued as seen by the survival of a small fraction (~14%) of RanBPM isoform mutants flies to adulthood (Supplementary Table S1 and S3). These flies displayed a spread wing phenotype similar to that of Dichaete (D) mutants also seen in all escapers of heteroallelic combinations that supported survival to adulthood.

The elav gene and the elav-GAL4 line used in this study are highly expressed in postmitotic neurons of the central and peripheral nervous system [52] and transiently expressed in glia and neuroblasts [53]. Therefore, it is possible that expression in cells other than differentiated neurons contributed to the observed rescue of the mutant phenotypes. Targeted expression of either RanBPM isoform using the glia-specific driver repo-GAL4 did not rescue any of the phenotypes (data not shown) and co-localization with the anti-Repo antibody was not observed (Figure S5 G-L). However, the inability to rescue a given mutant phenotype when using a heterologous promoter may be due to target gene expression that is below the threshold required, rather than inappropriate tissue-specific expression. Taken together, our results demonstrate a role in larval behavior, for RanBPM gene function in the nervous system, perhaps in postmitotic neurons, and suggest that the lack of RanBPM gene function in this tissue contributes to the observed lethality.

Expression of RanBPM in the Kenyon neurons of the MB is sufficient to rescue the response to light and feeding phenotypes

Given that the long isoform of RanBPM is highly expressed in the MB neurons, we asked whether expression in these neurons was sufficient to rescue RanBPM mutant phenotypes. To that end, we used GAL4 drivers whose expression overlapped in the MB. 247-GAL4 is derived from an enhancer found in the Dme2 promoter and is highly and nearly exclusively expressed in the MB neurons throughout larval development until adulthood ([50] and data not shown). Dme2-GAL4 is expressed in several neurons, in addition to those of the MB, as well as in somatic muscles [49]. The pattern of expression of 386-GAL4 extends to numerous peptideergic neurons [31] and includes MB neurons (Figure S4 C-D’).

The performance of RanBPM isoform mutants expressing either isoform under the regulation of MB GAL4 drivers in the ON/OFF assay, is shown in Figure 5 panel A. Targeted expression of at least one of the two RanBPM isoforms under the regulation of two (247 and Dme2) of the three MB GAL4 drivers was sufficient to restore the response index of RanBPM mutants to levels that are not statistically different from that of control strains. Targeted expression of the long isoform using the 386-GAL4 driver markedly improved the response of mutants but this was not statistically significant. In the case of the 247-GAL4 driver, marked and significant rescue occurred using either isoform when larvae

Nervous system expression of either RanBPM isoforms rescues all RanBPM larval behavioral phenotypes

In order to identify the cells or group(s) of cells in which RanBPM expression plays a role in larval behavior, we generated RanBPM constructs whose expression can be targeted to particular cells and tissues under the control of tissue-specific GAL4 transgenic constructs [56]. Using standard genetic crosses we created homozygous RanBPM isoform mutant flies carrying the RanBPM isoform under the control of the GAL4-responsive DNA binding site (UAS) as well as different GAL4 drivers.

Figure 3. RanBPM function is required for cell proliferation. Confocal micrographs of larval brains immunolabeled with anti-phosphoH3, detected with Alexa 488 secondary antibody. Images shown are projected Z-stacks of 10–13 (wild type) or 5–7 sections (RanBPM isoform) sections at 2 μm intervals. The RanBPM mutant CNS (A) is smaller than the wild type control (B) and show reduced phosphoH3 labeling, indicating that lack of RanBPM gene function disrupts proliferation.

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with phosphoH3 immuno-labeling (Figure S5A–F). Similarly, RanBPM immuno-labeling did not co-localize with that of the glial marker, repo (Figure S5G-L), [54]. In the CNS, RanBPM labeling appeared to be cytoplasmic but not restricted to the neuronal cell bodics (e.g. Figure 4G–I). We did not detect RanBPM labeling in the neuromuscular junction (NMJ) [28]. Some, but not all motoneurons labeled by the motoneuron reporter D42-Gal4 [55], also expressed RanBPM (data not shown).

In the brain hemispheres RanBPM was highly, but not exclusively expressed in a bilateral dorsal cluster of neurons. This labeling co-localized with the expression of a mushroom body marker (247-GAL4;UAS-C77-GFP, [50]; Figure 4A–F). At this level of resolution it appeared that all RanBPM-expressing cells present in this dorsal cluster co-expressed the mushroom body-specific element 247-GAL4. While we did not detect RanBPM expression in the imaginal discs, we did find expression in the muscle fibers attached to the mouth hooks and in the cytoplasm of the ring gland (data not shown and insert in Figure 4A). This pattern of expression was not detected in RanBPM mutant larvae, demonstrating that it is not due to cross reactivity of this antibody (Figure 4J and K). Moreover, targeted expression of the long isoform in the mutant background restored RanBPM expression, as detected by immunolabelling with this antibody, in a pattern consistent with that of the GAL4 driver employed (Figure 4D–E). No signal was detected in wild type larval brain specimens when we used an antibody generated against a portion of the RanBPM open reading frame common to both proteins (kindly provided by Paul Lasko, McGill University [8]). Therefore, we do not know whether in the larval CNS expression of the short isoform differs from that of the long isoform. Nevertheless, as described below, targeted rescue experiments indicate that these two isoforms are functionally equivalent.
were grown at 29°C, the optimal temperature for the function of the yeast transcription factor *GAL4*.

In rescued larvae (*RanBPM*<sup>K05201</sup>; *247:RanBPM<sup>long</sup>), expression of the long isoform of *RanBPM* was restored in the MB neurons (Figure 4L). The subcellular localization within the MB structure however, differs from that of wild type samples. In the rescued specimens *RanBPM* immunolabelling is found in the cell bodies, the calyx and in the pedunculi, while in wild type specimens it is restricted to the cell bodies (Figure 4 compare A to L). This is likely due to the heterologous promoter, in this case the *247-GAL4* driver, driving the expression of the *UAS-RanBPM* target construct to levels above that of wild type, thereby causing the ectopic expression of this protein in the pedunculi.

Significant improvement of locomotion occurred in mutants expressing the long isoform in the MB neurons when grown at 29°C (*RanBPM*<sup>K05201</sup>; *247:RanBPM<sup>long</sup>), Figure 5B). While marked increase in locomotion was also observed when the other MB *GAL4* drivers were used, they were not statistically significant. Lethality was marginally rescued by expression of *RanBPM<sup>long</sup>* under the control of *Dmef2-GAL4* and not at all when *247-GAL4* and *386-GAL4* were employed (Table S1 and S3). In contrast feeding was completely restored when all three MB *GAL4* drivers

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**Figure 4. RanBPM<sup>long</sup> is highly expressed in the Kenyon cells of the mushroom body.** Confocal micrographs of wild type larval CNSs immunolabeled with anti-*RanBPM<sup>long</sup>* detected by Cy3-conjugated secondary antibody (red) and carrying a MB reporter construct *247-GAL4/UAS-CD8-GFP* (green, *247:GFP, A–F*) or double labeled with a pan neuronal antibody (anti-<em>elav</em>, green, *G–I*). *RanBPM* labeling is found in the ventral cord (arrowhead in A), the ring glands (inset in A) and in two bilateral clusters of neurons located in the brain hemispheres (box in A–C). Boxed area in A–C is magnified in D–F. These neurons are the Kenyon cells of the MB as seen by extensive co-localization with the MB reporter *247-GFP*. In the abdominal portion of the ventral cord, *RanBPM* expression is found widely but not at the same level in all cell bodies (arrow in G and H). CNS dissected from homozygous mutant larvae (*J, RanBPM<sup>K05201</sup>; K, RanBPM<sup>S135</sup>*, and the *RanBPM<sup>K05201</sup>* mutant expressing the long isoform under the regulation of the MB specific driver *247-GAL4* (*L, RanBPM<sup>K05201</sup>; 247:RanBPM<sup>long</sup>*) were labeled with anti-*RanBPM* antibody (red) that recognizes the long isoform. The CNSs of larvae homozygous for the two most severe alleles show reduction in the volume of the CNS and absence of the characteristic *RanBPM* expression pattern in the brain (*J and K*). In the rescued sample (*L, RanBPM<sup>K05201</sup>; 247:RanBPM<sup>long</sup>*), *RanBPM* immunolabelling is restricted to the MB cell bodies and neuropil, the latter represents ectopic expression due to the high level of expression of this *GAL4* driver (compare A with L).

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Figure 5. Targeted expression of RanBPM in subsets of neurons rescues the control of locomotion by light and the locomotion phenotypes of RanBPM<sup>65201</sup> mutants. A - The presence of any of the GAL4 drivers or either one of the UAS constructs did not significantly change the larval response to light. Pan-neural (elav-GAL4) expression of either RanBPM<sup>long</sup> or RanBPM<sup>short</sup> constructs significantly increased the response to light of RanBPM<sup>65201</sup> mutants relative to that of RanBPM<sup>65201</sup> mutants alone or carrying one copy of either one of the GAL4 drivers or UAS constructs. Expression of the short RanBPM isoform under the control of 247-GAL4 (29 C) and Dmef2-GAL4, significantly increased the response to light phenotype of RanBPM<sup>65201</sup> mutants. RanBPM mutant larvae expressing the UAS-RanBPM<sup>short</sup> construct in the MB under the control of the 247-GAL4 driver displayed significantly higher response to light only when grown at 29 C. * p < 0.05 N ≥ 10 (ANOVA: F(10.082) = 20,256, p < 0.001). B - Locomotion of RanBPM<sup>65201</sup> mutant larvae expressing the long or short isoform under the control of various GAL4 drivers is shown as the number of pixels (mean ± SEM) in 30 sec of the assay. Locomotion was significantly reduced in RanBPM<sup>65201</sup> mutant larvae relative to revertant control and did not improve significantly when these mutants carried one copy of any of the GAL4 drivers or one copy of the long isoform construct. Expression of the long RanBPM isoform under the control of elav-Gal4 and 247-GAL4 (29 C) significantly increased locomotion above that of all mutant controls to levels still below that of the RanBPM<sup>65201</sup> revertant control. Presence of the short RanBPM isoform construct significantly increased the locomotion of RanBPM<sup>65201</sup> mutant larvae and that was not increased when a GAL4 driver was introduced. * p < 0.05, N ≥ 11 (ANOVA: F(19,60) = 20,257, p < 0.001). doi:10.1371 journal.pone.0010652.g005

were employed (Table 1). We concluded that RanBPM expression in the Kenyon neurons is sufficient to rescue the behavioral phenotypes of RanBPM mutants.

**RanBPM** gene function contributes to FMRP-dependent processes

*RanBPM* was previously identified in a yeast two-hybrid screen as a putative partner of FMRP [18]. FMRP is an RNA binding protein implicated in the transport, translational control and metabolism of mRNAs encoding proteins involved in synaptic plasticity [reviewed by [23,57,58]]. The *Drosophila* orthologue, *dfmr1*, has been used extensively as a model for FMRP function in the regulation of synaptic structure and function [reviewed by [59]]. *dfmr1* mutants display over-elaboration of neuronal structure exemplified by midline crossing of intrinsic MB neurons as well as increased branching and bouton number at the larval neuromuscular junction (NMJ) [20,21,32,60]. Moreover *dfmr1* function is required for activity dependent pruning of MB axons [24].

We asked whether *RanBPM* function is required for FMRP-dependent processes. To that end, we conducted genetic epistasis analysis to determine whether *dfmr1* mutant phenotypes are modified (enhanced or suppressed) by changes in the level of *RanBPM* function. *RanBPM*<sup>65201</sup> mutations cause lethality during late larval development while the *dfmr1* mutant alleles used here, *dfmr1<sup>14055</sup>* and *dfmr1<sup>14564</sup>* cause lethality at the pharate adult stage and are hypomorphic and amorphic mutations respectively. Interestingly *RanBPM;dfmr1* double homozygotes die during embryogenesis. We asked whether reduction in *RanBPM* function modifies the NMJ overgrowth phenotype of *dfmr1* mutants. *dfmr1* mutant NMJ is characterized by pronounced synaptic overgrowth as seen by nearly 50% increase in branching and in the total number of boutons ([32,60]; and Figure 6). While the NMJ of double mutants does not appear entirely normal, introduction of one copy of the lack of function allele *RanBPM*<sup>65201</sup> is sufficient to suppress the overgrowth NMJ phenotype. Increased branching and bouton number is reduced in these double mutants to levels comparable to that of wild type controls. These observations indicate that *RanBPM* function is required for the expression of *dfmr1* phenotype and suggest that *RanBPM* and *dfmr1* function converge in the mechanisms underlying synaptic growth.

**Discussion**

The *RanBPM* gene was first identified in vertebrates and proposed to function as a scaffolding protein [reviewed in [61]]. *RanBPM* mutations in vertebrate model systems are not yet available. Therefore, its precise role in these processes remains to
be established. The first reported mutational approach to RanBPM function was in Drosophila. In this model organism, RanBPM was shown to regulate the development of the female germ line niche [8].

We report a novel role for RanBPM in larval behavior. RanBPM mutant larvae showed normal growth and behavior until the late second instar stage. Soon after the last molt, growth and food intake ceased. Our results suggest that this is due to suppression of the food seeking behavior characteristic of this stage. Mutant larvae moved away from the food, displaying precocious wandering, a hallmark behavior of the late third instar stage (Figure 2 and Movie S2). Moreover, light-induced changes to locomotion were nearly abolished, and these larvae displayed sluggish and uncoordinated locomotion (Figure 1).

Consistent with the high level expression of RanBPM in the MB neurons we found that targeted expression in these cells was sufficient to restore light-induced changes in locomotion and the feeding phenotypes to nearly wild type levels (Figure 5 and Table 1). Lethality was partially rescued when the elav-GAL4 or the Dmef2-GAL4 driver were used (Table 1). The latter [Dmef2-GAL4 driver] is expressed in CNS neurons that include MB Kenyon cells as well as somatic muscles. Rescue of lethality was not achieved when the 247-GALA driver, a selective MB marker, was used (Table S3). Therefore, it is not clear whether the suppression of feeding phenotype is the sole cause for the recessive lethality caused in RanBPM mutations.

Rhythmic behaviors such as larval locomotion, are generated by neuronal networks called central pattern generators (CPG). Sensory input provided by the multidendritic sensory (MD) neurons is essential to coordinate the rhythmic peristalsis that constitutes the larval forward movement [62,63]. Therefore, the current model for the coordination of larval movement demands the proper development and synchronized function of four groups of cells: neurons that constitute the CPG, MD neurons, motorneurons and body wall muscles. The uncoordinated phenotype of RanBPM mutants may be due to lack of gene function in any of these cell types. Collectively, the GAL4 drivers employed in our studies are expressed in all of these different cell types. However while CNS expression, in particular in the MB, neurons was sufficient to restore the ability of RanBPM mutant larvae to display the characteristic light-induced changes in locomotion, none restored locomotion to levels similar to that of control strains. It is possible that the GAL4 drivers employed did not provide an adequate level and/or timing of RanBPM expression that reached the threshold required. Additional experiments are required in order to explore the role of RanBPM function in the various cellular components that contribute to this rhythmic behavior and in particular the function of MB function in the control of larval locomotion.

Extensive evidence has accumulated pointing to the MB, in adult flies, as the site of olfactory learning [64]. Additional roles for MB neurons in adult behavior include regulation of motor activity, centropobia, habit formation and saliency-based decision-making [65,66,67,68]. In the Drosophila larvae the role of MB neurons has not been as extensively investigated.

MB function has not been directly implicated in Drosophila feeding behaviour. Interestingly the Drosophila peptide sNPF, which is structurally related to the mammalian neuropeptide Y (NPY), a regulator of food consumption, is expressed in larvae and adults, in a large subset of MB intrinsic neurons and in neurons located in the ventral cord [47,48,69]. Ubiquitous expression of dsRNA constructs targeting sNPF mRNA reduced food intake in Drosophila larvae and adults [69]. Whether sNPF expression in MB neurons is implicated in this phenotype is yet to be established. Expression of sNPF in RanBPM mutants appeared normal at the level of resolution afforded by immunolabelling and confocal microscopy. Given the large number of cells labeled by sNPF antibody a clonal approach must be used in order to evaluate the projection of individual neurons in a RanBPM mutant background.

We found that while the size of RanBPM mutant larvae is reduced, cell fate and differentiation appeared normal (Figures S2 and S4). Smaller larval size is reflected in the nervous system, as a marked reduction in the number of actively dividing cells. Interestingly, in RanBPM mutants, mitotically-active cells are nearly absent in the abdominal portion of the ventral cord while more anteriorly, in the thoracic portion and brain hemispheres,
proliferation is markedly reduced but not absent (Figure 3). This phenotype is similar to that reported for newly-hatched larvae allowed to feed for 1 day and kept in amino acid-free medium for three days thereafter. It reflects the anterior to posterior wave of re-entry of neuroblasts into the cell cycle triggered by the first feeding of the newly-hatched Drosophila larva [70].

Impaired cell proliferation in RanBPM mutants is a non-autonomous phenotype. RanBPM expression in the nervous system was not detected in mitotically-active cells. Targeted expression of RanBPM in a subset of the CNS neurons rescued the reduced larval size and reduced CNS proliferation phenotypes. These observations are consistent with the notion that, in RanBPM mutants, reduced size and cell proliferation may be due to precocious cessation of foraging and food ingestion with consequent starvation. Starvation in turn, leads to the reduction of cell proliferation in the nervous system and reduced larval size due to inhibition of endoreplication and larval cell growth.

pumpless (ppl) and klumpfuss (klu) mutants display suppression of food-seeking behavior and reduced size similar to that observed in RanBPM mutants but move vigorously and coordinately suggesting that disruption of locomotion detected in RanBPM mutants is not necessarily due to lack of food intake [3,42]. ppl encodes a subunit of the amino acid glycine cleavage system and is highly expressed in the fat body [42]. In contrast, klu, encodes zinc finger containing transcription factor, which is widely expressed in the nervous system and is involved in the regulation of proneural proteins [3,71].

The current model postulates the existence of humoral mitotic signals detected by the fat body and regulated by amino acid ingestion, which may also regulate larval feeding behavior [42,70]. Amino acid ingestion triggers the secretion, by the fat body, of the Drosophila Acid-Labile Subunit (dALS). Binding of dALS to Drosophila Insulin-Like Peptides (DILPs) in turn regulates their bioavailability [72]. Hyperactivation of insulin receptor/phosphoinositide 3-kinase (Inr/P13K) signaling in the fat body regulates their bioavailability [72]. Hyperactivation of insulin receptor/phosphoinositide 3-kinase (Inr/P13K) signaling in the fat body induces larval wandering –like behavior as seen by a food dispersal phenotype [73], reviewed by [74] and[75]. The focus of the suppression of larval feeding and reduced cell proliferation phenotype in RanBPM mutants is in the nervous system. Therefore it is not unreasonable to suggest that RanBPM mutants may be deficient in the reception and/or transduction of humoral signals, perhaps derived from the fat body, that trigger and sustain larval foraging behavior in order to ensure adequate food intake. The finding that feeding behaviour is rescued in RanBPM mutants in which RanBPM function is selectively restored to the MB neurons suggests that this structure plays a role in feeding behaviour (Table 1).

Several studies report direct binding of RanBPM to a number of different proteins (e.g. [11,12,13,14,15,16,17,76,77] reviewed by [61]). However the functional relevance of several of these interactions is yet to be established. One of the potential partners of RanBPM function, as established in protein binding assays, is the RNA binding protein FMRP [18]. Lack of FMRP function is the underlying cause of the more prevalent form of inherited mental retardation [19]. FMRP function is highly conserved and is required for mRNA transport and translational suppression in the context of synaptic plasticity. In Drosophila as well as in other model systems, lack of dfmr1 function promotes synaptic elaboration[20,21,22,32].

As a first step toward the identification of signaling pathways or biochemical functions impacted by RanBPM loss, we carried out a genetic epistasis experiment between RanBPM mutation and mutations of the Drosophila orthologue of FMRP, the dfmr1 gene. The finding that reduction in RanBPM function suppresses the NMJ overgrowth phenotype of dfmr1 mutants suggest that RanBPM contributes to FMRP dependent processes (Figure 6).

Our experiments did not address whether this is a direct or indirect contribution. It is possible that in the context of NMJ development, RanBPM functions as a component of a protein complex, that positively regulates dfmr1 function as an inhibitor of translation. Thus, reduction of RanBPM relieves the remaining dfmr1 function thereby partially suppressing the NMJ overgrowth. In our studies we used dfmr1 mutations that, while causing similar NMJ phenotypes, were either a complete loss (dfmr1A535A), or a partial loss of function (dfmr1P3517). Yet, the RanBPM[k05201] mutation suppressed the dfmr1 NMJ phenotype to the same extent (Figure 6). These observations suggest, as a more likely model, that RanBPM function is required in a dose-dependent fashion for NMJ development and not directly for dfmr1 function. Taken together, the work reported here support the hypothesis that the underlying cause of the RanBPM behavioral phenotypes is lack of gene function in the MB neurons, and point to a novel role for this structure in larval behavior.

Supporting Information

Figure S1 Locomotion of RanBPM[k05201] mutant and RanBPM revertant in constant dark. Representative perimeter stacks generated using DIAS depicting larval locomotion during 60 sec in the absence of light transition under safelight. Found at: doi:10.1371/journal.pone.0010652.s001 (2.69 MB TIF)

Figure S2 RanBPM[k05201] third instar foraging larvae are smaller than control larvae. DIAS was used to measure the long axis of larval images. Under the DIAS function “measure” we used “simple length” to measure the number of pixels along the anterior posterior axis of individual larvae. The “scale” function was used to obtain the scale factor value employed to convert pixels into μm. RanBPM[k05201] mutants are significantly smaller than all control larvae of the same developmental stage. yw is significantly smaller than OR but not RanBPM revertant, *p<0.05, N=10, (ANOVA, F(3, 36) = 54.943, p<0.0001). Found at: doi:10.1371/journal.pone.0010652.s002 (6.03 MB TIF)

Figure S3 Contact chemosensory assay. The assay arena was divided into four quadrants. Opposing quadrants were filled with 1% agar in 1 M NaCl or in water. Larvae were placed in the center and allowed to migrate. Their distribution was determined at 10 and 15 min. Larvae that did not migrate more than 1 cm from the center of the plate were not included. The percentage of larvae present in the non salt quadrants was plotted. All genotypes showed a non-random distribution between the salt non-salt quadrants. The preference of RanBPM mutants for the non-salt quadrant at 10 min (χ² = 2.70, DF = 3, p<0.439) and 15 min (χ² = 3.84, DF = 3, p<0.279) is not significantly different from that of the control genotypes (OR, yw, RanBPM revertant), N>50. Found at: doi:10.1371/journal.pone.0010652.s003 (3.03 MB TIF)

Figure S4 RanBPM is not required for differentiation and/or maintenance of various larval neurons. Confocal micrographs of RanBPM[k05201] larval brains labeled with various reporters and antibodies. In all panels the symbol ’ ′ (prime) indicates homozygous RanBPM[k05201] larval brains labeled with various reporters and antibodies. In all panels the symbol ’ ′ (prime) indicates homozygous RanBPM[k05201] larval brains labeled with various reporters and antibodies. In all panels the symbol ’ ′ (prime) indicates homozygous RanBPM[k05201] larval brains labeled with various reporters and antibodies. In all panels the symbol ’ ′ (prime) indicates homozygous RanBPM[k05201] larval brains labeled with various reporters and antibodies.
commonly used to label the MB neuropil area indicates that MB structure in these mutants is largely unaltered at this level of resolution but the volume may be reduced (red, D, D'). The FMRF amide antibody detects a subset of FMRF amide like peptides that contain a common RF amide sequence on their C-terminal. Included in this group is sNPF, the only known peptide to be expressed in the Kenyon cells. The expected pattern of expression detected by FMRF amide antibody is seen in the whole CNS (E–F'), MB neuropil area (F, F') and Kenyon cells (G, G'). 5-HT labeling reveals a stereotyped segmental pattern of neuronal cell bodies in RanBPM mutant, indistinguishable from control (I and I'), however cell counts revealed a small but significant reduction in the cell number (Table in J). Consistent with the observation that the MB neuropil area is reduced in these mutants we found that the 5HT arborization particularly found in the larval optic neuropil is reduced in RanBPM[K05201] mutants (arrowhead in I and I'). All images except for those shown in panels B and B' are projections of Z stacks of 20 sections at 1 to 2 μm intervals.

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Figure S5 RanBPM is not expressed in proliferating cells or glia. Confocal micrographs of third instar larval CNS double labeled with anti-RanBPM (green) and anti-phosphoH3 (red, A–F), or the glial marker anti-Repo (red, G–L). Boxed areas in B and K are magnified in D–F and J–K respectively and highlight RanBPM expression in the area of the lobes where the MB neurons are located. Co-localization was not detected for anti-RanBPM (green) and anti-phosphoH3 (red, A–F), or the glial marker anti-Repo (red, G–L). Included in this group is sNPF, the only known peptide to be expressed in the Kenyon cells. The expected pattern of expression detected by FMRF amide antibody is seen in the whole CNS (E–F'), MB neuropil area (F, F') and Kenyon cells (G, G'). 5-HT labeling reveals a stereotyped segmental pattern of neuronal cell bodies in RanBPM mutant, indistinguishable from control (I and I'), however cell counts revealed a small but significant reduction in the cell number (Table in J). Consistent with the observation that the MB neuropil area is reduced in these mutants we found that the 5HT arborization particularly found in the larval optic neuropil is reduced in RanBPM[K05201] mutants (arrowhead in I and I'). All images except for those shown in panels B and B' are projections of Z stacks of 20 sections at 1 to 2 μm intervals.

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Table S1 Lethality complementation test for RanBPM mutants. Found at: doi:10.1371/journal.pone.0010652.s005 (7.04 MB TIF)

Table S2 Fraction of RanBPM mutant larvae that ingested food in 30 min.

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