**Drosophila Mon1 and Rab7 interact to regulate glutamate receptor levels at the neuromuscular junction**

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**ABSTRACT** Regulation of post-synaptic receptors plays an important role in determining synaptic strength and plasticity. The *Drosophila* larval neuromuscular junction (nmj) has been used extensively as a model to understand some of these processes. In this context, we are interested in the role of *Drosophila* Monensin sensitivity protein 1 (DMon1) in regulating glutamate receptor (GluRIIA) levels at the nmj. DMon1 is an evolutionarily conserved protein which, in complex with calcium caffeine zinc sensitivity1 (CCZ1), regulates the conversion of early endosomes to late endosomes through recruitment of Rab7. C-terminal deletion mutants of Dmon1 (Dmon1D181) exhibit lethality. The escapers have a short life span and exhibit severe motor defects. At the nmj, these mutants show defects in synaptic morphology and a strong increase in GluRIIA levels. The mechanism by which Dmon1 regulates GluRIIA is unclear. In this study, we have characterized an EMS mutant referred to as pog and demonstrate it to be an allele of Dmon1. Further, we have examined the role of rab7 in regulating GluRIIA. We show that similar to Dmon1, knock-down of rab7 using RNAi in neurons, but not muscles, leads to an increase in GluRIIA. Loss of one copy each of Dmon1 and rab7 leads to a synergistic increase in receptor expression. Further, overexpression of an activated Rab7 can rescue the GluRIIA phenotype observed in Dmon1D181 mutants. Together, these results highlight a neuronal role for Rab7 in GluRIIA regulation and underscore the importance of the endo-lysosomal pathway in this process.

**KEY WORDS:** Dmon1, Rab7, GluRIIA, nmj, Drosophila

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**Introduction**

Post-synaptic neurotransmitter receptors play an important role in determining synaptic strength and plasticity. Receptor levels are altered in response to changes in neurotransmitter release to maintain homeostasis. The *Drosophila* larval neuromuscular junction (nmj) has been used for a long time as a model to study synaptic development and homeostasis. These synapses are glutamatergic akin to the central synapses found in the vertebrate CNS. The glutamate receptors at the nmj are tetramers made up of four subunits: GluRIIA or GluRIIB, GluRIIC, GluRIID and GluRIIE. Receptors carrying the A or the B type subunit differ in their conductance properties and are shown to be reciprocally regulated such that an increase in the levels of GluRIIA leads to corresponding decrease in GluRIIB (Petersen et al., 1997; DiAntonio et al., 1999; Marrus et al., 2004). The mechanisms that regulate glutamate receptor expression, clustering and turn-over are still poorly understood. A classic study by Broadie and Bate more than two decades ago showed that expression of GluRIIA during embryogenesis takes place in two waves. The first wave is autonomous to the muscle and independent of innervation, while the second wave is dependent of innervation. Clustering of the receptors is also dependent on contact between the nerves and muscle (Brodie and Bate., 1993). In the absence of innervation or electrical activity, the muscles fail to upregulate receptor expression in the second wave and clustering fails (Brodie and Bate., 1993). A more recent analysis of the kinetics of GluRIIA transcription suggests that most of the mRNA required for protein synthesis during the...
embryonic and larval stages is transcribed during embryogenesis with a sharp increase in transcript levels occurring around the time of nerve-muscle contact. An equally sharp decrease in mRNA levels is seen soon after, with no further increase in mRNA levels. The change in GluRIIA protein levels appears to be more gradual and steadily increases through embryonic and larval stages (Ganesan et al., 2009) suggesting that most of the mRNA synthesized during embryogenesis for use during larval stages is sequestered as ribonucleoparticles for subsequent use.

The molecular nature of the pre-synapticinputs that regulate post-synaptic receptor expression, clustering and maintenance of these GluRIIA clusters has remained elusive. Recently, Lola was identified as a transcription factor regulating the expression of many post-synaptic components including GluRIIA. Interestingly, Lola was found to be and sensitive to neuronal activity, with increase in stimulation leading to downregulation of Lola activity (Fukui et al., 2012). The molecular identity of other signals, if any, that might function as part of this pre-synaptic regulatory network are not known.

*Drosophila* Mon1 (Dmon1) is a conserved endocytic factor which, in complex with CC21, helps recruit Rab7 onto endosomes, facilitating the conversion of an early endosome to a late endosome. The protein was first identified in yeast as a factor required for all fusion events to the lysosome (Wang et al., 2002; Wang et al., 2003). This function of recruiting Rab7 appears to be conserved across species from yeast to mammals including plants (Poteryaev et al., 2010; Nordmann et al., 2010; Kinchen and Ravichandran., 2010; Yousefian et al., 2013; Cui et al., 2014). While the cellular function of Mon1 is now well established, its physiological role is only beginning to be addressed. For example, in Arabidopsis, mon1 mutants show poor male fertility due to delayed tapetal degeneration and programmed cell death (Cui et al., 2017). In Cryptococcus neoformans Mon1 is essential for virulence (Son et al., 2018).

We had previously generated, through P-element excision, a mutation in *Dmon1* (Dmon1*156*), that deletes the C-terminal region of the protein. *Dmon1*181 mutants show poor viability and motor abilities. The synaptic morphology in these animals is altered. However, a striking phenotype observed in these animals was the elevated levels of GluRIIA in synaptic, and often, the extrasynaptic regions. The requirement of *Dmon1* appears to be primarily neuronal, with neuronal knock-down phenocopying the GluRIIA phenotype and expression of *Dmon1* in neurons but not neuronal, being able to rescue the mutant lethality (Deivasigamani et al., 2015). The mechanism by which DMon1 regulates GluRIIA levels is not clear. Given the relationship between DMon1 and Rab7, we wondered whether Rab7 might play a role in this process. In this study, we have focused our attention to determining whether DMon1 and Rab7 interact to regulate GluRIIA levels at the larval neuromuscular junction.

Here, we describe a new allele of *Dmon1* previously identified and referred to as pgo (Matthew et al., 2009). Using genetics and sequencing, we demonstrate that pgo (hereafter referred to as *Dmon1*156) is an allele of *Dmon1* with a stop codon at residue 157 of the amino acid sequence. Like the C-terminal deletion mutant, *Dmon1*156/181 adults show impaired motor abilities and shortened lifespan. At the synapse these mutants show an increase in GluRIIA levels. To evaluate the role of Rab7 in the regulation of GluRIIA, we have knocked down rab7 in neurons using RNAi. Interestingly, while neuronal knock-down leads to an increase in the intensity of GluRIIA staining, downregulation of rab7 in the muscle has little effect. Further, there appears to be a dose dependent effect with Rab7C: high levels of expression leads to an increase in GluRIIA.

We show that *Dmon1* and rab7 interact to regulate GluRIIA levels. Transheterozygous mutants carrying one copy each of the *Dmon1*156 and rab7 mutation show an increase in GluRIIA levels that are comparable to homozygous *Dmon1*181. Furthermore, expression of Rab7C in a *Dmon1*181 mutant background is able to suppress the GluRIIA phenotype suggesting that the two genes are likely to be part of the same regulatory pathway. Our results thus demonstrate a role for a presynaptic DMon1-Rab7 dependent endosomal pathway in regulating post-synaptic receptor levels.

### Results

**Identification and characterization of Dmon1156**

pgo is an EMS mutant first described as a mutation affecting germband extension during gastrulation in early embryogenesis (Matthew et al., 2009). pgo is homozygous lethal and the mutants fail to survive beyond the second instar larval stage. It shows non-complementation with *Df(2L)9062* indicating that the mutation is likely to be in either *Dmon1* or *smog* - two genes that are uncovered by the deficiency line. We crossed pgo to *Dmon1*181 and *Dmon1*129 mutants: the former carries a deletion restricted to the 3’ region of *Dmon1*, while the latter has a deletion spanning the 3’ region of *Dmon1* and 5’ region of the adjacent smog gene (Deivasigamani et al., 2015). Non-complementation was observed in both cases indicating that the mutation is likely to be in *Dmon1*. Indeed, sequence analysis of *Dmon1* in pgo showed presence of single base pair change leading to an amber mutation at residue 157 (Fig. 1A), suggesting absence of a full-length protein. We hereafter refer to pgo as *Dmon1*156.

We have previously shown that homozygous *Dmon1*181 escaper adults have a short lifespan and strong motor defects. Consistent with this, both *Dmon1*156/161 and *Dmon1*156/156/*Df(2L)9062* animals were short-lived and showed poor motor abilities (Fig. 1 B,C). To determine if neuronal expression of *UAS-Dmon1::HA* rescued these defects, we expressed the transgene in *Dmon1*181 mutants and found that both defects could be rescued completely to match wildtype animals (Fig.1 B,C). Interestingly, the lethality in *Dmon1*181 mutants could not be rescued by expression of *UAS-Dmon1::HA* indicating the presence of a second site ‘hit’ that contributes to lethality in these mutants. However, the rescue of lethality and climbing defect in *Dmon1*156/181 through expression of *UAS-Dmon1::HA* supports and validates *Dmon1*156 as an allele of *Dmon1*.

**Characterization of synaptic phenotypes in Dmon1156**

Homozygous *Dmon1*156/181 mutant larvae show defects in synaptic morphology: the boutons tend to be larger and odd shaped with many supernumerary or satellite boutons (Fig 2 B,C; Deivasigamani et al., 2015). A similar phenotype was observed in *Dmon1*156/181 larvae: the boutons were often bigger and, like the other allele, showed many more supernumerary boutons (Fig. 2 D,E) with no change in bouton number.

Homozygous *Dmon1*181/181 mutants show elevated levels of GluRIIA at post-synaptic densities. On an average, the observed increase is nearly two-fold (Deivasigamani et al., 2015). An increase in GluRIIA levels was also observed in *Dmon1*156/181 animals (Fig. 2 H-H’).
However, unlike Dmon\(^{1^{156}}\) the increase was approximately 30% (Fig. 2I). The phenotypes associated with synaptic morphology and GluRIIA were suppressed upon expression of UAS-Dmon1::HA, confirming that they are caused due to loss of Dmon1 function (Fig. 2 F,J). This provides further validation to Dmon\(^{1^{156}}\) being an allele of Dmon1.

Loss of Dmon1 does not alter quantal size or quantal content

Overexpression of GluRIIA increases post-synaptic sensitivity leading to increase in quantal size, which is the response to release of a single neurotransmitter vesicle. These animals also show an increase in the evoked response or evoked junction potential (EJP) but no change in quantal content or the total number of vesicles released (Petersen et al., 1997).

Homozygous Dmon\(^{1^{156}}\) mutants show nearly a 2-fold increase in GluRIIA levels. To determine whether the increase in receptor levels has similar physiological effects as GluRIIA overexpression, we carried out intracellular recordings on homozygous Dmon\(^{1^{156}}\) animals. Consistent with the increase in GluRIIA, in both genotypes, a significant increase in EJP was observed (FIG. 3B-C & H) which was rescued by neuronal expression of UAS-Dmon1::HA (Fig. 3 E,H). Surprisingly, we failed to observe any change in quantal size, frequency of the mEPSPs (Fig. 3 F,G) and quantal content (Fig. 3I). The lack of an increase in post-synaptic sensitivity suggests that either many of the GluRIIA positive receptors are non-functional or, that there is compensation possibly due to the decrease in vesicle size and/or the decrease in GluRIIB which has been observed in these mutants (Deivasigamani et al., 2015).

Neuronal knock-down of Rab7 increases GluRIIA

The above results show that loss of Dmon1 leading to an increase in GluRIIA levels alters neurotransmission. However, the mechanism by which DMon1 regulates receptor levels is not clear. Given that the conserved function of DMon1 is to recruit Rab7, we sought to determine whether the regulation of GluRIIA is dependent

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**Fig. 1. Identification and characterization of the Dmon\(^{1^{156}}\) allele.** (A) Shown is the amber mutation in Dmon1 that generates a stop codon at position 157 of the amino acid sequence. (B) Lifespan of different allelic combinations of Dmon1\(^{156}\) mutants. The strongest allelic combination Dmon1\(^{156}/D\) shows a half-life of approximately 17-18 days. Neuronal expression of UAS-Dmon1::HA rescues life-span to wild-type levels. (C) Shown is a graph of the climbing speed of wildtype and Dmon1\(^{156}\) mutants flies at 1, 5 and 8 days post-eclosion. Motor abilities are compromised in Dmon1\(^{156}/D\) and Dmon1\(^{156}/Df(2L)9062\) animals. Consistent with the increase in GluRIIA, in both genotypes, a significant increase in EJP was observed (FIG. 3B-C & H) which was rescued by neuronal expression of UAS-Dmon1::HA (Fig. 3 E,H). Surprisingly, we failed to observe any change in quantal size, frequency of the mEPSPs (Fig. 3 F,G) and quantal content (Fig. 3I). The lack of an increase in post-synaptic sensitivity suggests that either many of the GluRIIA positive receptors are non-functional or, that there is compensation possibly due to the decrease in vesicle size and/or the decrease in GluRIIB which has been observed in these mutants (Deivasigamani et al., 2015).
on Rab7. As a first step, we studied the localization of Rab7 at the neuromuscular junction using a genome engineered Rab7<sup>42FLP</sup> line (Dunst et al., 2015) by staining these larvae with anti-HRP and anti-GFP antibodies. Numerous GFP positive puncta were seen distributed all over the muscle and also near boutons in the peri-synaptic regions (Fig. 3J). Faint GFP positive puncta were also detected inside boutons suggesting presence of late endosomes in the pre-synaptic compartment (Fig. 3J inset). The puncta were completely absent in Dmon<sup>1<sup>181</sup></sup> mutants further reconfirming the role of Dmon1 in recruiting Rab7 onto vesicles (Fig. 3K).

Next, we checked whether knock-down of rab7 in neurons alters GluRIIA levels. Expression of UAS-rab7RNAi using D42-GAL4 resulted in a 40% increase in the intensity of GluRIIA staining (Fig. 3 O,P). A near two-fold increase in intensity was observed upon expression of the RNAi using a double GAL4 line namely (Fig. 3 O,P). A near two-fold increase in intensity was observed (Fig. 4A). Notably, Dmon<sup>1<sup>181</sup></sup> and rab7<sup>174</sup> larvae did not show any significant change in synaptic GluRIIA levels when compared with wildtype (Fig. 4C). Further, the near two-fold increase in GluRIIA intensity in trans-heterozygous Dmon<sup>1<sup>181</sup></sup> and rab7<sup>174</sup> larvae alters the expression or localization of GluRIIA.

Curiously, expression of UAS-rab7RNAi using C57-GAL4 and mhc-GAL4 had little effect on GlurIIA levels (Supplementary Figure S1).

Based on the above results, we checked if expression of a constitutively active Rab7 (Rab7<sup>G12V</sup>) leads to a decrease in GlurIIA levels. Interestingly, the effect appeared to be the opposite and dose dependent: while overexpression with D42-GAL4 did not result in any significant change in GlurIIA staining, an increase in receptor levels was observed with C155-GAL4; elav-GAL4 and OK6-GAL4 lines (Fig. 3P).

Mon1 and Rab7 interact to regulate GlurIIA

The above results indicate that rab7 dependent regulation of GlurIIA is primarily pre-synaptic. To determine whether Dmon1 and rab7 interact to regulate GlurIIA, we examined GlurIIA levels in the trans-heterozygous mutant larvae carrying one copy each of Dmon<sup>1<sup>181</sup></sup> and rab7<sup>174</sup>. A significant increase in receptor expression was observed (Fig. 4B). Notably, Dmon<sup>1<sup>181</sup></sup> and rab7<sup>174</sup> larvae did not show any significant change in synaptic GlurIIA levels when compared with wildtype (Fig. 4C). Further, the near two-fold increase in GlurIIA intensity in trans-heterozygous Dmon<sup>1<sup>181</sup></sup> and rab7<sup>174</sup> larvae alters the expression or localization of GlurIIA. Curiously, expression of UAS-rab7RNAi using C57-GAL4 and mhc-GAL4 had little effect on GlurIIA levels (Supplementary Figure S1).

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**Fig. 2. Characterization of the synaptic phenotype in Dmon<sup>1<sup>TM4</sup></sup>**

(A-D) Muscle 4 synapse stained with HRP (red) and Dlg (green). (A) Wildtype (w<sup>1118</sup>) synapse. Note the pearl-on-a-string arrangement of boutons. (B,C) Synapse of a homozygous Dmon<sup>1<sup>181</sup></sup>; D42-GAL4 (B) and Dmon<sup>1<sup>181</sup></sup>; D42-GAL4/+; Df(2L)9062 (C) larva. Note presence of fused and irregularly shaped boutons (asterisk) and satellite boutons (arrowheads). (D) Dmon<sup>1<sup>156/181</sup></sup> larvae exhibit a similar synaptic morphology with irregularly shaped boutons and satellite or supernumerary boutons. (E) Quantification of the average number of satellite boutons per synapse in different alleles of Dmon1. A significant increase in satellite boutons is seen in the mutants (w<sup>1118</sup>; 2.3±0.286, n=19; homozygous Dmon<sup>1<sup>181</sup></sup>; 7.89±0.54, n=19; Dmon<sup>1<sup>156/181</sup></sup>; 5.5±0.6, n=18; Dmon<sup>1<sup>156</sup></sup>; Df(2L)9062: 4.504±0.562, n=16). (F) The satellite bouton phenotype in Dmon<sup>1<sup>156/181</sup></sup> is suppressed by expression of UAS-Dmon1::HA (elav-GAL4/+; Dmon<sup>1<sup>156/181</sup></sup>; elav-GAL4; Dmon<sup>1<sup>156/181</sup></sup>; UAS-Dmon1::HA; n=22). (G,H) A muscle 4 synapse from w<sup>1118</sup> (G) and Dmon<sup>1<sup>156/181</sup></sup> (H) stained with HRP (red) and GlurIIA (green). Note the increase in intensity of GlurIIA. (I) Quantification of the intensity of GlurIIA in wildtype (1±0.056, n=16), homozygous Dmon<sup>1<sup>181</sup></sup> (1.74±0.085, n=17) and Dmon<sup>1<sup>156/181</sup></sup> (1.29±0.065, n=20) animals. The latter shows a 30% increase in GlurIIA intensity compared to a near 70% increase in homozygous Dmon<sup>1<sup>181</sup></sup> mutants: 1±0.047 (elav-GAL4; Dmon<sup>1<sup>156/181</sup></sup>; n=24) vs. 0.58±0.042 (elav-GAL4; Dmon<sup>1<sup>156/181</sup></sup>; UAS-Dmon1::HA; n=22). *** indicates P<0.0001; ** indicates P<0.001; * indicates P<0.01; n, number of synapses.
Fig. 3. Neuronal downregulation of rab7 increases GluRIIA levels. (A-E) Representative traces of evoked (Red) and spontaneous (Black) responses of indicated genotypes. Recordings were carried out in 1.5 mM Ca\(^{2+}\) containing HL3 and nerves innervating muscle 6/7 were stimulated at 1 Hz to record evoked responses. (F) Average mEJP amplitude of control (w\(^{1118}\), 0.77±0.05 mV), mutants (homozygous Dmon1\(^{∆181}\)/Dm\(^{1118}\)/Df(2L)9062, 0.70±0.05 mV), mutants (homozygous Dmon1\(^{∆181}\)/Dm\(^{1118}\)/Df(2L)9062;UAS-Dmon1::HA, 0.80±0.03 mV). (G) Average mEJP frequency of control (w\(^{1118}\), 2.59±0.16 Hz), mutants (homozygous Dmon1\(^{∆181}\)/Dm\(^{1118}\)/Df(2L)9062, 2.82±0.18 Hz) and rescued mutants (C155-GAL4/+; Dmon1\(^{∆181}\)/Df(2L)9062;UAS-Dmon1::HA, 3.04±0.19 Hz). (H) Average EJP amplitude of control (w\(^{1118}\), 43.47±0.08 mV), mutants (homozygous Dmon1\(^{∆181}\)/Dm\(^{1118}\)/Df(2L)9062, 48.64±1.17 mV), mutants (homozygous Dmon1\(^{∆181}\)/Dm\(^{1118}\)/Df(2L)9062, 49.12±1.33 mV), control for rescue (C155-GAL4/+; Dmon1\(^{∆181}\)/Df(2L)9062;UAS-Dmon1::HA, 44.78±1.31 mV). The EPSP amplitudes reveal significantly enhanced evoked potentials in Dmon1 alleles, which are rescued by expression of UAS-Dmon1::HA in the mutants. (I) Average quantal content of control (w\(^{1118}\), 58.97±3.45), mutants (homozygous Dmon1\(^{∆181}\), 62.67±3.76), mutants (homozygous Dmon1\(^{∆181}\)/Df(2L)9062, 69.46±5.07), rescued animals (C155-GAL4/+; Dmon1\(^{∆181}\)/Df(2L)9062, 69.70±4.66) and rescued animals (C155-GAL4/+; Dmon1\(^{∆181}\)/Df(2L)9062, 69.70±4.66). Quantal content was obtained by dividing average EJP amplitude with average mEJP amplitude of individual recordings. (J,K) Rab7\(^{EGFP}\) line stained with anti-HRP and anti-GFP. (L-O) Representative images of the effect of downregulation of rab7 on GluRIIA using D42-GAL4 (L,M) and OK6-GAL4 (N,O). Note the increase in receptor levels (M and O, respectively). (P) Change in staining intensity of GluRIIA upon expression of UAS-rab7RNAi and UAS-rab7\(^{CA}\) with different drivers: D42-GAL4: Control (D42-GAL4/+; 1 ± 0.056, n=14) vs. UAS-rab7RNAi (D42-GAL4>UAS-rab7RNAi, 1.43±0.119, n=13) vs. UAS-rab7\(^{CA}\) (D42-GAL4>UAS-rab7\(^{CA}\), 1.62±0.134, n=12); C155-GAL4: Control (C155-GAL4/+; elav-GAL4/+, 1 ± 0.099, n=10) vs. UAS-rab7RNAi (C155-GAL4>UAS-rab7RNAi, 1.78±0.072, n=20) vs. UAS-rab7\(^{CA}\) (C155-GAL4>UAS-rab7\(^{CA}\), 1.60±0.12, n=19). *** indicates P<0.0001; ** indicates P<0.001; * indicates P<0.01; n, number of synapses. (F,G,H,I) The numbers in the bars represent number of animals used for recordings and/or quantification. Error bars represent standard error of the mean (SEM). Statistical analysis based on two-tailed Student’s t-test. * P<0.05, ** P<0.01. *** P<0.001.
rab7<sup>+/−</sup> mutants was comparable to the receptor levels observed in Dmon<sup>1<sup>181</sup></sup>, indicating that Dmon<sup>1</sup> and rab7 interact in a dose dependent manner to regulate GluRIA. The increase in receptor expression was observed not only at the synapse, but in extrasynaptic regions as well which is often seen in Dmon<sup>1</sup> mutants (Fig. 4B). A similar synergistic increase in receptor expression was also seen in Dmon<sup>1</sup>; rab7<sup>1/−</sup> animals (Fig. 4D).

It is to be noted that while the steep increase in receptor levels is comparable to Dmon<sup>1</sup> mutants, the synaptic morphology in these trans-heterozygous animals is very distinct: the boutons though bigger, are well-spaced with fewer satellites (Fig. 4B). To further confirm the genetic interaction between Dmon<sup>1</sup> and rab7 and to test if they function as part of the same pathway we expressed UAS-rab7<sup>C</sup>A in homozygous Dmon<sup>1</sup> mutants to determine if it reduced GluRIA levels. Indeed, a strong decrease in GluRIA staining was observed in these animals (Fig. 4F,G) with fewer number of satellite boutons (Fig. 4H). Together these results indicate that Dmon<sup>1</sup> and rab7 interact in the pre-synaptic compartment to regulate GluRIA at the larval neuromuscular junction.

**Fig. 4.** Dmon<sup>1</sup> and rab7 interact in a synergistic manner to regulate GluRIA levels. (A,B) Homozygous Dmon<sup>1<sup>181</sup></sup> (A) and Dmon<sup>1<sup>181</sup>/−; rab7<sup>1/−</sup> (B) stained with anti-HRP (red) and anti-GluRIA (green) show comparable levels of GluRIA. (C) Graph showing normalized values for intensity of GluRIA:HRP ratios. Control (w<sup>1118</sup>, 1±0.04, n=18); Dmon<sup>1<sup>181</sup>/− (1.23±0.104, n=24); rab7<sup>1/−</sup> (1.135±0.057, n=18); homozygous Dmon<sup>1<sup>181</sup></sup> (1.888±0.097, n=25); Dmon<sup>1<sup>181</sup>/−; rab7<sup>1/−</sup> (1.965±0.088, n=32). The increase in GluRIA in trans-heterozygous mutant animals is comparable to homozygous Dmon<sup>1<sup>181</sup></sup>. (D) Graph showing the normalized values for intensity of GluRIA:HRP ratios. Control (w<sup>1118</sup>, 1±0.06, n=20); Dmon<sup>1<sup>156</sup>/− (0.99±0.076, n=20); rab7<sup>1/−</sup> (0.88±0.091, n=20); Dmon<sup>1<sup>156</sup>/− (1.37±0.08, n=19); Dmon<sup>1<sup>181</sup></sup>; rab7<sup>1/−</sup> (1.777±0.09, n=19). (E,F) Expression of UAS-rab7<sup>C</sup>A in homozygous Dmon<sup>1</sup> mutants rescues the synaptic and GluRIA phenotype associated with the mutants. (G) Normalized values for intensity of GluRIA:HRP ratios. Mutant control (Dmon<sup>1<sup>181</sup>/−; elav-GAL4/+), 1±0.061, n=18); Rab7<sup>1/−</sup> ‘rescue’ (elav-GAL4/+; UAS-rab7<sup>C</sup>A, 0.515±0.041, n=20); Mutant control (OK6-GAL4/+; Dmon<sup>1<sup>181</sup>/−), 1±0.071, n=20); rab7<sup>1/−</sup> ‘rescue’ (OK6-GAL4/+; UAS-rab7<sup>C</sup>A, 0.685±0.061, n=20); (H) Expression of UAS-rab7<sup>C</sup>A rescues synaptic morphology. Fewer satellite boutons are seen. Mutant control (elav-GAL4/+; Dmon<sup>1<sup>181</sup>/−; OK6-GAL4/+), 5.6±0.42, n=15); Rab7<sup>1/−</sup> ‘rescue’ (elav-GAL4/+; Dmon<sup>1<sup>181</sup>/−; OK6-GAL4/+; UAS-rab7<sup>C</sup>A, 2.95±0.24, n=20); mutant control (OK6-GAL4/+; Dmon<sup>1<sup>156</sup>/−; OK6-GAL4/+; UAS-rab7<sup>C</sup>A, 6.8±0.28, n=20); rab7<sup>1/−</sup> ‘rescue’ (OK6-GAL4/+; Dmon<sup>1<sup>156</sup>/−; OK6-GAL4/+; UAS-rab7<sup>C</sup>A, 2.05±0.43, n=18). *** indicates P<0.0001, ** indicates P<0.001 and * indicates P<0.01. n, number of synapses.
Discussion

Rab proteins play a crucial role in regulating intracellular trafficking by directing endosomes to their appropriate destination. Their dysfunction is associated with various neurodegenerative diseases and cancers (Stenmark, H., 2009; Wandinger-Ness and Zerial., 2014; Banworth et al., 2018). Rab7 marks late endosomes and regulates the trafficking and fusion of vesicles with the lysosome. The processes regulated by Rab7 are diverse and range from retrograde trafficking, lysosome positioning, regulation of protein kinases through effectors etc many of which have implications in neurodegenerative disorders (Deinhart et al., 2006; Dodson et al., 2012; Stroupe, C., 2018). Mutations in Rab7 are associated with Charcot-Marie-Tooth disease 2b (CMT2B) — a type of neurodegenerative disorder that causes axonal degeneration (Verhoeven et al., 2003).

The Drosophila Mon1-Rab7 complex functions as a GEF for Rab7 and is essential for its recruitment onto endosomes (Nordmann et al., 2010; Poteryaev et al., 2010). Therefore, it is of interest to determine the extent to which the phenotypes associated with loss of Dmmon1 are dependent on rab7. This would be helpful in identifying and understanding Rab7-independent functions of Dmmon1, if any. We have addressed this in the context of the Drosophila neuromuscular junction where Dmmon1, through a pre-synaptic mechanism, regulates GluRIIA levels at the synapse.

We find that presynaptic knock-down of rab7 leads to a significant increase in GluRIIA levels which is comparable to that seen upon knock-down of Dmmon1. Further, trans-heterozygous mutants Dmmon1181/+; rab7+/− and Dmmon1181/+; rab7−/+ mutants show a dramatic increase in GluRIIA indicating that the two genes interact to regulate GluRIIA. Further, expression of a constitutively active Rab7 (Rab7CA) suppresses not only the defects in synaptic morphology but also the increase in GluRIIA in Dmmon1 mutants.

Our results indicate that neuronal and not muscle knock-down of rab7 leads to an increase in the intensity of GluRIIA staining. This is in variance with a study by Lee and colleagues (Lee et al., 2013) involving knock-down of tbc1D15-17 in Drosophila. However, TBC1D15 RNAi mediate knock-down of tbc1D15 in the muscle but not neuron is seen to affect GluRIIA levels. However, it is to be noted that the effect of rab7 RNAi and rab7CA on GluRIIA has not been evaluated in this study. Moreover, little is known about Rab7 regulation in Drosophila and it is possible that the GAP protein regulating Rab7 in the neuron and muscles is different. These questions will need to be addressed in future studies.

The results from overexpression of rab7 loss-of-function like phenotype. This will need to be tested. The mutation in Dmmon1 affects the conserved domain. The increase in intensity of GluRIIA in Dmmon1181/+; rab7−/+ is seen to approximately 30% compared to the nearly two-fold increase seen in Dmmon1181/+ mutants. We do not completely understand the reason for this variation amongst the alleles. It would be interesting to determine functional contribution of the different domains to regulation of GluRIIA levels.

At this point, the precise manner in which Dmmon1-Rab7 axis regulates GluRIIA is not clear. An interesting point to be noted in this context is that Dmmon1 is released from the pre-synaptic compartment (Deivasigamani et al., 2015). While we cannot completely rule out the possibility of Dmmon1 and Rab7 having independent effects on GluRIIA, the evidence based on the genetic interaction between the two, and localization of Dmmon1 suggests that the Dmmon1-Rab7 complex probably regulates a secretory process that influences GluRIIA levels. At the nmj, signaling molecules such as wingless and synaptotagmin-4 are known to be released via exosomes (Korkut et al., 2009; Korkut et al., 2013) which arise from multivesicular bodies or late endosomes. It would be interesting to test if the regulation of GluRIIA via the Dmmon1-Rab7 axis involves such a mechanism.

It is intriguing that post-synaptic knock-down of rab7 does not affect GluRIIA levels in any significant manner. This is somewhat unexpected since in mammalian synapses, endo-lysosomal pathways are known to control the trafficking and turn-over of AMPA receptors (Fernandez-Monreal et al., 2012; Haussler and Schlett, 2013). While the mechanisms regulating GluRIIA turnover at the Drosophila larval nmj are still poorly characterized, the absence of an effect with UAS-rab7RNAi would imply existence of independent pathways. The other possibility could be that knock-down of Rab7 in the muscle is insufficient to elicit a phenotype. These possibilities will need to be explored in greater detail. In summary, our findings here highlight a novel role for the neuronal endo-lysosomal pathway in regulating post-synaptic GluRIIA levels, the details of which will need to be elucidated in future studies.

Materials and Methods

Flystocks

All stocks were maintained on regular cornmeal agar medium. pog’ (Kind gift from S. Kerridge); Dmmon1181 (Deivasigamani et al., 2015). The following stocks were obtained from the Bloomington Stock Centre: UAS-Rab7RNAi (#27051); UAS-Rab7Q67L::YFP (#9779); D42-GAL4 (Rab7 EYFP (#62545); D42-GAL4 (#8816); OK6-GAL4 (#64199). Rab7 (Kind gift from the Juhasz lab; Hegedus et al., 2016.)

Molecular mapping and behavioral characterization of Dmmon1

Dmmon1181 previously referred to as pog’ (Matthew et al., 2009), does not complement Dmmon1181 indicating that the mutation is likely in Dmmon1. To identify the EMS mutation, we PCR amplified and sequenced the entire Dmmon1 gene from Dmmon1181. A single base pair change (C to T) at position 4851219 of the flybase sequence was results in an amber mutation (CAG to TAG) at position 157 of the amino acid sequence of Dmmon1. The lifespan and motor assays to characterize Dmmon1181 phenotype was carried out as described in Deivasigamani et al., 2015.

Immunohistochemistry and imaging

Larval fillets were fixed with Bouins for 15 minutes at room temperature (23-24°C) and stained using standard protocols (Patel et al., 1994).
2% BSA was used for blocking. Anti-HRP (Sigma, 1:1000); anti-GluRIIA (concentrate; DSHB, Iowa state, 1:200); anti-GFP (Thermo Fisher, 1:1000). Imaging was done using a Leica SP8 confocal system. All images were captured with a 63X, 1.4 N.A. objective. Image analysis was carried out using ImageJ (NIH) or Fiji software. For measurement of GluRIIA intensity, all genotypes of a set were stained simultaneously. Each time, a cocktail of the primary and secondary antibodies was made and distributed equally to all the genotypes. Imaging was carried out using identical conditions of gain and laser power. Intensity measurements for GluRIIA were carried out as described in Menon et al., 2004 and Deivasigamani et al., 2015.

**Electrophysiology**

Intracellular electrophysiology recordings were performed as described previously (Chodhury et al., 2016). Briefly, third instar wandering larvae were dissected in modified HL3 saline containing 70 mM NaCl, 5 mM KCl, 20 mM MgCl2, 10 mM NaHCO3, 115 mM sucrose, 5 mM trehalose, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 1 mM EGTA at pH 7.2. For recordings the EGTA was replaced with 1.5 mM CaCl2 in modified HL3 saline (Verstreken et al., 2002). mEPSPs were recorded for 60 s in absence of any stimulation. For evoked responses (EPPs), motor axons were stimulated at 1 Hz and responses were recorded for 1 minute. All the recordings were made using sharp glass microelectrodes with 15-25 MQ resistance from muscle 6 of A2 hemi-segment. Data was analysed using an offline software minianalysis (Synaptosoft).

Statistical Analysis. Analysis was done using GraphPad Prism software. Two-tailed student’s t-test and ANOVA was used for analysis. Graph in Fig. 4D represents pooled data from 3 independent experiments. Each set contained all genotypes, stained simultaneously and imaged using identical conditions. All values represented in the figures are mean ± s.e.m.

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