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The microRNA miR-1 regulates a MEF-2 dependent retrograde signal at neuromuscular junctions

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Summary

We show that miR-1, a conserved muscle specific microRNA, regulates aspects of both pre- and post-synaptic function at *C. elegans* neuromuscular junctions. miR-1 regulates the expression level of two nicotinic acetylcholine receptor (nAChR) subunits (UNC-29 and UNC-63), thereby altering muscle sensitivity to acetylcholine (ACh). miR-1 also regulates the muscle transcription factor MEF-2, which results in altered pre-synaptic ACh secretion, suggesting that MEF-2 activity in muscles controls a retrograde signal. The effect of the MEF-2-dependent retrograde signal on secretion is mediated by the synaptic vesicle protein RAB-3. Finally, acute activation of levamisole-sensitive nAChRs stimulates MEF-2-dependent transcriptional responses, and induces the MEF-2-dependent retrograde signal. We propose that miR-1 refines synaptic function by coupling changes in muscle activity to changes in pre-synaptic function.

Keywords

microRNA; miR-1; synapse; nAChR; nicotinic receptors; acetylcholine; MEF2; MEF-2; RAB-3; Rab3; retrograde; synaptic transmission; C. elegans

Introduction

Faithful synaptic transmission requires that neurotransmitter release is matched to post-synaptic receptor function. This coordination is achieved through bidirectional signaling between pre- and post-synaptic cells. A variety of mechanisms are proposed to mediate coupling of pre- and post-synaptic function including signaling by retrograde messengers and trans-synaptic adhesion molecules (Davis and Bezprozvanny, 2001; Kalinovsky and Scheiffele, 2004; Scheiffele, 2003). By bridging the pre- and post-synapse, these molecules are considered likely candidates for coordinating synapse refinement. Many questions remain to be answered about this process. What upstream signaling pathways are directly involved in

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coupling changes across the synapse? What downstream molecular targets mediate the pre- and post-synaptic changes? Here we provide evidence that a conserved muscle microRNA, miR-1, is involved in this process.

MicroRNAs regulate several aspects of neuronal development (Kosik, 2006). In *C. elegans*, left/right asymmetric cell fate of chemosensory neurons during development is controlled by microRNAs (Johnston and Hobert, 2003), as is the switch to neuronal-specific gene expression during vertebrate neurogenesis (Cao et al., 2006). In zebrafish, a single microRNA is required for development of the nervous system (Giraldez et al., 2005). While microRNAs were originally identified based on their regulation of developmental processes, it is becoming apparent that microRNAs also regulate properties of mature tissues. For example, in cultured rodent neurons, microRNAs have been implicated in coupling the response to neurotrophins with changes in the size of dendritic spines (Schratt et al., 2006).

The early lethality of genetic mutants in the microRNA biosynthetic pathway and the limited availability of conditional knockouts of individual microRNAs has prevented detailed analysis of post-developmental roles for microRNAs such as miR-1. The sequence and muscle-specific expression of miR-1 are conserved in all species examined (Nguyen and Frasch, 2006). Fly and mouse miR-1 knockouts are lethal (Sokol and Ambros, 2005; Zhao et al., 2007), underscoring its importance as a developmental regulator. *C. elegans mir-1* mutants are viable, allowing the dissection of miR-1 function in adult animals. We show that miR-1 regulates synaptic transmission at neuromuscular junctions, and that it does so by regulating nicotinic acetylcholine receptors (nAChR) and the generation of a retrograde signal that modulates the function of pre-synaptic terminals.

Results

The *C. elegans* genome contains a single miR-1 ortholog with perfect sequence conservation (Lee and Ambros, 2001). To analyze the expression of *mir-1*, we generated a transcriptional reporter construct in which the *mir-1* promoter drives expression of GFP. This construct expressed GFP in both pharyngeal and body muscles, with no apparent expression in other tissues, including neurons (Fig. 1A). Thus, miR-1 expression is restricted to muscles in flies, mice, and worms. Two independent *mir-1* deletion mutants (*gk276* and *tm1635*) were viable as homozygotes and did not display any overt behavioral abnormalities. Muscle cell numbers and morphology were superficially normal in both *mir-1* mutants (data not shown). These results suggest that lack of miR-1 did not grossly alter muscle development in worms.

Decreased muscle responsiveness to nicotinic agonists in *mir-1* mutants

Worm body muscle contracts in response to activation of nicotinic acetylcholine receptors (nAChRs). Body muscles express two classes of nAChRs (Richmond and Jorgensen, 1999) (Fig. 1B). Levamisole-sensitive nAChRs (LevRs) are hetero-pentamers containing alternative α-subunits (UNC-38, UNC-63, and LEV-8) and non-α-subunits (UNC-29 and LEV-1) (Brown et al., 2006). Body muscles also express ACR-16/α7 homo-pentamers, which are insensitive to levamisole (Francis et al., 2005; Touroutine et al., 2005). To determine if some aspect of muscle function was altered, we assayed the sensitivity of *mir-1* mutants to levamisole. Levamisole binds to and activates LevRs, leading to muscle contraction and subsequent paralysis. Homozygous *mir-1(gk276)* and *mir-1(tm1635)* mutants were both resistant to the paralytic effects of levamisole (Fig. 1C, data not shown). Consistent with these behavioral results, levamisole-evoked currents recorded from body wall muscles were significantly reduced in both *mir-1* mutants (Fig. 1D, E). The function of ACR-16 channels can be assayed by measuring ACh-evoked currents in *unc-29* mutants (Francis et al., 2005; Touroutine et al., 2005). The Ach-evoked currents in *unc-29* single mutants and *unc-29 mir-1* double mutants were indistinguishable (Fig. 1F, G). These results suggest that the number or activity of LevRs

on the surface of body muscles was decreased in *mir-1* mutants, whereas ACR-16 receptors were unaffected.

Agonist-evoked currents are likely to be mediated by both synaptic and non-synaptic nAChRs. To determine if miR-1 regulates the function of synaptic receptors, we recorded excitatory post-synaptic currents (EPSCs) produced by the endogenous activity of motor neurons. We observed a 23% decrease in the amplitude of endogenous EPSCs in *mir-1* mutants (Fig. 1H, I, p=0.002, Student's t-test), as would be predicted if loss of miR-1 reduces the activity of synaptic nAChRs

Approximately 80% of the excitatory post-synaptic current in body muscles is carried by ACR-16/α7 homo-pentamers, with LevRs mediating the remaining 20% (Francis et al., 2005; Touroutine et al., 2005). Consequently, the decreased endogenous EPSC amplitudes observed in *mir-1* mutants could result from a change in either the LevRs, ACR-16 receptors, or both. To distinguish between these possibilities, we recorded EPSCs from double mutants lacking miR-1 and one of the two classes of nAChRs. The amplitude of endogenous EPSCs in *acr-16*; *mir-1* double mutants and in *unc-29 mir-1* double mutants were significantly lower than those observed in the corresponding single mutants, *acr-16* and *unc-29* respectively (Supplementary Figs. 1 and 2) These results suggest that miR-1 regulates the activity of synaptic ACR-16 and LevRs.

Different classes of nAChRs can often be distinguished by their kinetics. Consistent with this idea, we found that the decay kinetics of endogenous EPSCs in *unc-29* mutants were significantly faster than those recorded from wild type controls (Supplementary Fig. 2). These results indicate that synaptic currents mediated by ACR-16 receptors have faster decay kinetics than those mediated by LevRs. Similarly, in *mir-1* mutants, the decay kinetics of endogenous EPSCs were significantly faster than those recorded from wild-type controls, and were virtually identical to those observed in *unc-29* mutants (Fig. 1, Supplementary Fig. 2). These data are consistent with a miR-1-dependent change in the activity of synaptic LevRs. Interestingly, EPSC decay time constants in *mir-1 unc-29* double mutants were faster than in *unc-29* single mutants (Supplementary Fig. 2), suggesting that the kinetics of ACR-16-mediated synaptic currents are also affected in *mir-1*. These results suggest that miR-1 regulates sensitivity of body muscles to ACh.

miR-1 regulates nAChR subunit abundance

The decreased function of LevRs in *mir-1* mutants could be caused by altered abundance of receptor subunits. Consistent with this idea, we found predicted miR-1 binding sites in the 3'UTRs of the *unc-29* and *unc-63* mRNAs (Fig. 2A). These putative miR-1 binding sites were conserved in the orthologous genes in the nematode *C. briggsae* (Supplementary Fig. 3). The bioinformatic algorithm mirBase also identified *unc-63* as a predicted miR-1 target (Griffiths-Jones et al., 2006). By contrast, miR-1 binding sites were not found in the 3'UTRs of mRNAs encoding other muscle nicotinic receptor subunits, including UNC-38, LEV-8, LEV-1, and ACR-16.

We next investigated whether miR-1 regulates *unc-29* and *unc-63*. First, we analyzed expression of endogenous subunits by immunostaining (Fig. 2B–D) and immunoblotting (Fig. 2E, F). In both assays, we observed increased abundance of endogenous UNC-29 and UNC-63 subunits in *mir-1* mutants, whereas the abundance of a control subunit (UNC-38) was unaltered. To determine if regulation of UNC-29 abundance was mediated by the predicted miR-1 binding sites in the 3'UTR, we made a reporter construct in which the *unc-29* 3'UTR was appended to GFP. When this construct was expressed in body muscles, we observed increased GFP abundance in *mir-1* mutants compared to wild type (Fig. 2G, H). By contrast, GFP abundance was unaltered in *mir-1* mutants for reporter constructs containing the *unc-38* 3'UTR, or a

mutagenized *unc-29* 3'UTR in which nucleotides in the three predicted miR-1 binding sites were scrambled (Fig. 2G, H). These results support the idea that the *unc-29* and *unc-63* mRNAs are miR-1 targets, while the *unc-38* mRNA is not.

Increased UNC-29 and UNC-63 expression decreases muscle sensitivity to levamisole

Decreased LevR function in *mir-1* mutants could be caused by increased abundance of UNC-29 and UNC-63, or by misregulation of other miR-1 targets. To distinguish between these possibilities, we analyzed the effect of increasing expression of these subunits in wild-type animals. *unc-29* and *unc-63* cDNAs were fused to the *unc-54* 3'UTR, which lacks predicted miR-1 binding sites. When the UNC-29 and UNC-63 constructs were co-expressed in body muscles of wild-type animals, we observed resistance to the paralytic effects of levamisole (Fig. 3A), and a significant decrease in the amplitude of levamisole-evoked currents recorded from body muscles (Fig. 3B, C). By contrast, no change in levamisole sensitivity was observed when either construct was expressed alone in wild-type animals, nor when both constructs were co-expressed in *mir-1* mutants (Fig. 3A). Thus the levamisole resistance phenotype of *mir-1* mutants can be explained by the coordinate up-regulation of UNC-29 and UNC-63. Over-expression of UNC-29 and UNC-63 was not sufficient to cause a change in either the amplitude or kinetics of endogenous EPSCs (Supplementary Fig. 4), suggesting that other miR-1 targets may contribute to these defects. These results are consistent with prior studies showing that changes in subunit composition alter the kinetics, conductance, and agonist affinity of mammalian nAChRs (Millar, 2003).

Synaptic transmission is reduced in *mir-1* mutants

The decreased muscle responsiveness to ACh prompted us to consider whether miR-1 regulates synaptic transmission at NMJs. Steady-state acetylcholine (ACh) secretion in living worms can be assayed by measuring resistance to the acetylcholine esterase inhibitor aldicarb. Aldicarb treatment causes accumulation of ACh at neuromuscular junctions (NMJs), leading to acute paralysis. Mutations that decrease ACh secretion confer resistance to aldicarb-induced paralysis (Miller et al., 1996). We found that *mir-1* mutants were resistant to aldicarb (Fig. 4C), as would be predicted if ACh release had been decreased. To more directly assay ACh release, we recorded EPSCs from body muscles. We found that the EPSC amplitudes and the total synaptic charge transfer evoked by a depolarizing stimulus were significantly decreased in *mir-1* mutants (Fig. 4A, B; Supplementary Table 1).

In principle, altered EPSCs and aldicarb responses could arise from either a change in pre-synaptic release of ACh, or from the decreased activity of muscle LevRs. We did several experiments to distinguish between these possibilities. First, the reduction in EPSC amplitude in *mir-1* mutants (55%) was significantly greater than that observed in *unc-29* mutants (20%) (Francis et al., 2005). Second, to control for changes in muscle sensitivity, we measured quantal content. Changes in muscle sensitivity to ACh alters quantal size, i.e. charge transfer that occurs following release of a single synaptic vesicle. The number of vesicles released following a stimulus can be estimated by measuring the quantal content, which measures the ratio of post-synaptic charge transfer that occurs following a depolarizing stimulus to that which occurs during an endogenous EPSC. We found that quantal content was also significantly reduced in *mir-1* mutants (Supplementary Table 1). These results suggest that the decreased stimulus-evoked EPSC observed in *mir-1* mutants is unlikely to result from a change in muscle sensitivity.

The pool of primed synaptic vesicles can be measured by evoking fusion with hypertonic sucrose (Rosenmund and Stevens, 1996). We found that the EPSC evoked by hypertonic sucrose was not altered in *mir-1* mutants compared to wild-type controls (Fig. 4D, E),

suggesting that the decreased ACh secretion observed in *mir-1* mutants was not caused by a change in vesicle priming.

As an independent assay of pre-synaptic function, we also recorded endogenous EPSCs. The rate of endogenous EPSCs was significantly reduced in *mir-1* mutants (~50% wild-type rate, $p<0.0001$, Student's t-test) compared to wild-type controls (Fig. 4G–I). The decreased endogenous EPSC rate was not a secondary consequence of the decreased EPSC amplitude (Fig. 1H), because only a 19% change in EPSC rate would be predicted to result from a 23% decrease in amplitude. These results suggest that *mir-1* mutants have a pre-synaptic defect leading to decreased ACh secretion.

Synapse density is not altered in *mir-1* mutants

The secretion defects observed in *mir-1* mutants might be caused by a decrease in the number of cholinergic NMJs. To address this possibility, we examined the localization of two active zone proteins, SYD-2 / α-Liprin (GFP-tagged), UNC-10 / RIM1 (endogenous protein visualized with anti-UNC-10 antibodies), and a synaptic vesicle protein (SNB-1 / Synaptobrevin, GFP-tagged) (Koushika et al., 2001; Zhen and Jin, 1999). We found that all three pre-synaptic proteins had similar distributions in wild-type and *mir-1* mutants. In particular, the densities of SYD-2 and SNB-1 puncta in wild-type and *mir-1* mutants were indistinguishable (Supplementary Fig. 5). We also examined the distribution of a post-synaptic protein, GFP-tagged ACR-16, and found that puncta density was not reduced in *mir-1* mutants (Wild type: 3.5 +/− 0.1 puncta/10μm, *mir-1*: 3.6 +/− 0.1 puncta/10μm, $p= 0.59$ Student's t-test). Consequently, loss of miR-1 did not grossly alter the distribution of pre- and post-synaptic proteins, nor the apparent number of cholinergic NMJs.

Interestingly, the fluorescence intensities of SYD-2 and SNB-1 were significantly altered in *mir-1* mutants: SNB-1 fluorescence decreased (20%, $p< 0.001$, Student's t-test), whereas SYD-2 fluorescence increased (39%, $p<0.0001$, Student's t-test). These changes in fluorescence are unlikely to be caused by changes in transcription, since opposite changes in fluorescence were observed despite the fact that both transgenes utilized the same promoter (*unc-129*). The altered abundance of SNB-1 and SYD-2 provides further evidence that *mir-1* mutants have pre-synaptic defects.

MEF-2 mediates the pre-synaptic function of miR-1

What miR-1 target mediates the pre-synaptic defects observed in *mir-1* mutants? The rate of endogenous EPSCs was not significantly altered in transgenic animals driving over-expression of UNC-29 and UNC-63 in body muscles (Supplementary Fig. 4), indicating that the pre-synaptic defect was not caused by changes in the abundance of nAChR subunits in muscles. LevRs are expressed both in muscle and neurons (Brown et al., 2006); however, *unc-29* mutants have a normal rate of endogenous EPSCs [Wild type (n=9) 11.4 +/− 2 Hz, 19.7 +/− 2.5 pA; *unc-29(x29)* (n=7) 13.3 +/− 2.2 Hz, 19.8 +/− 5 pA]. Thus, the pre-synaptic effects of miR-1 are unlikely to be mediated by altered function of pre-synaptic LevRs.

Since changes in UNC-29 and UNC-63 function cannot account for the observed decrease in ACh secretion, we searched for other miR-1 targets that might mediate this change in secretion. Members of the Mef2 family of transcription factors were recently shown to regulate formation of excitatory synapses in cultured rodent neurons (Flavell et al., 2006; Shalizi et al., 2006). This prompted us to investigate whether MEF-2 might function downstream of miR-1 in regulating pre-synaptic function. There is a single *mef-2* ortholog in *C. elegans* which is ubiquitously expressed, and mutants lacking MEF-2 are viable (Dichoso et al., 2000). We identified two miR-1 binding sites in the 3'UTR of the *mef-2* mRNA, both of which were conserved in *C. briggsae* (Supplementary Fig. 6). In addition, *mef-2* was identified as a

predicted miR-1 target in a genome-wide computational prediction of microRNA targets (Lall et al., 2006). The 3'UTR of the mouse Mef2a mRNA was previously shown to inhibit translation, and we found that it also contains three putative miR-1 binding sites (Supplementary Fig. 7) (Black et al., 1997).

These data suggest that the *mef-2* might be regulated by miR-1. Consistent with this idea, expression of a GFP reporter construct containing the *mef-2* 3'UTR in body muscles was significantly increased in *mir-1* mutants (Supplementary Fig. 6) whereas GFP levels were unaltered when this construct was expressed in motor neurons (Wild-type: 78.7 +/− 0.9 A.U, *mir-1*: 83.0 +/− 7.0 A.U., p= 0.58 Student's t-test). Mouse Mef2 promotes expression of miR-1 (Rao et al., 2006; Zhao et al., 2005); however, we found no evidence for MEF-2-dependent changes in the expression of a miR-1 transcriptional reporter (data not shown).

To determine if MEF-2 mediates the pre-synaptic functions of miR-1, we analyzed *mir-1 mef-2* double mutants (Fig. 5). Single mutants lacking MEF-2 had a 26% decrease in stimulus-evoked EPSC amplitude (p=0.02, Student's t-test) (Fig. 5A, B) and a normal rate of endogenous EPSCs (Fig. 5C–E). The effects of the *mir-1* mutation on stimulus-evoked EPSC amplitude and endogenous EPSC rate were eliminated in *mir-1 mef-2* double mutants, and in both cases the *mir-1* mutant defects were restored by transgenic expression of MEF-2 in body muscles (Fig. 5A–G). By contrast, the *mef-2* mutation did not correct the decreased levamisole-evoked current in *mir-1* mutants (Supplementary Fig. 8), nor did it correct the defect in endogenous EPSC decay rate (Fig. 5E, data not shown). However, the defect in endogenous EPSC amplitude was corrected in *mir-1 mef-2* double mutants. These results suggest that muscle expression of MEF-2 mediates all of the presynaptic effects of miR-1, but does not mediate many of its post-synaptic effects. These results further suggest that miR-1 and MEF-2 act in body muscles to control a retrograde signal that regulates pre-synaptic ACh secretion.

RAB-3 mediates the pre-synaptic function of miR-1 and MEF-2

The pre-synaptic secretion defect observed in *mir-1* mutants was associated with a dramatic increase in the synaptic abundance of YFP-tagged RAB-3. The increased YFP::RAB-3 abundance was eliminated in *mir-1 mef-2* double mutants, and was restored by transgenes driving *mef-2* expression in body muscles (Fig. 6A–F). RAB-3 is a small GTPase that associates with synaptic vesicles in a GTP-dependent manner (Sudhof, 2004). Decreased secretion can result from either increased or decreased RAB-3 expression (Schluter et al., 2004; Thiagarajan et al., 2004). This prompted us to test whether the decreased secretion observed in *mir-1* mutants was caused by a decrease in RAB-3 activity. If this were the case, one would expect that the secretion defects observed in *mir-1* and *rab-3* single mutants would be very similar, and that these defects would not be additive in *mir-1; rab-3* double mutants. Consistent with this idea, we found that stimulus-evoked EPSC amplitudes (Fig. 6G, H), and the rates of endogenous EPSCs (Fig. 6I, J) were indistinguishable in *mir-1* and *rab-3* single mutants, and additive effects were not observed in *mir-1; rab-3* double mutants (Fig. 6G–L). These results support the idea that the pre-synaptic defects observed in *mir-1* mutants are caused by decreased RAB-3 activity.

Levamisole treatment induces MEF-2-dependent changes in transcription

The transcriptional activity of mouse Mef2 is regulated by neuronal activity (Flavell et al., 2006; Shalizi et al., 2006). If MEF-2 transcriptional activity were similarly activity-dependent, the miR-1/LevR/MEF-2 pathway described here could provide a mechanism to couple changes in muscle activity to changes in pre-synaptic properties. To test this idea, we developed assays for MEF-2-dependent transcriptional activity. We identified several genes whose mRNA abundance is MEF-2-dependent, using gene chip analysis (D.S., K.T-P, and J.K., unpublished observations). We focused our analysis on the muscle-expressed gene *frm-4* (Roy et al.,

2002), whose expression was decreased 2.8 fold in *mef-2* mutants relative to wild type controls ($p<0.001$, Moderated t-statistic). Although *frm-4* may not represent a direct transcriptional target of MEF-2, the abundance of its transcript provides a molecular assay for MEF-2 activity.

To determine if MEF-2 transcriptional activity is regulated by LevRs, we analyzed the abundance of *frm-4* transcripts following a one hour application of levamisole. Levamisole treatment increased *frm-4* mRNA abundance in wild-type animals but had no effect in *unc-29* mutants (Fig. 7A), demonstrating that the increased *frm-4* expression was not caused by a non-specific effect of drug treatment. By contrast, following levamisole treatment of *mef-2* mutants, *frm-4* expression was not increased but was instead significantly decreased. These results suggest that activation of LevRs induces the transcriptional activity of MEF-2, and likely other transcription factors.

Levamisole treatment induces the MEF-2-dependent increase in RAB-3

If increased muscle activity, caused by levamisole treatment, induces the transcriptional activity of MEF-2, then we would expect that levamisole treatment would also induce the MEF-2-dependent retrograde signal. Consistent with this idea, we found that the pre-synaptic abundance of YFP::RAB-3 was significantly increased following treatment with levamisole (Fig. 7B,C,J). We did several controls to determine if this effect was specific for activation of LevRs. First, treatment with nicotine, which activates ACR-16 receptors (Francis et al., 2005), did not alter pre-synaptic RAB-3 fluorescence (Fig. 7P,Q). Second, the levamisole effect was dependent upon the activity of endogenous LevRs, as levamisole treatment of *unc-29* mutants did not alter RAB-3 fluorescence (Fig 7D,E,J). Third, this effect also required MEF-2 activity in muscles, since levamisole treatment of *mef-2* mutants also failed to alter RAB-3 levels, but transgenes driving MEF-2 expression in body muscles restored levamisole-induced increases in RAB-3 (Fig. 7F–J). Thus, acute activation of LevRs induces both MEF-2 transcriptional activity and the retrograde message, whereas acute activation of ACR-16 receptors (with nicotine) did not.

If LevR activity is required to induce the retrograde signal, we would also expect mutations inactivating these receptors would block the pre-synaptic effects of miR-1. Consistent with this idea, the effect of the *mir-1* mutation on pre-synaptic RAB-3 levels was eliminated in *mir-1 unc-29* double mutants (Fig. 7K–O). These results demonstrate that the activity of LevRs plays a pivotal role in regulating the activity of the retrograde message, mediating both acute induction following levamisole treatment and chronic induction in *mir-1* mutants.

Discussion

We show that the conserved microRNA miR-1 acts in *C. elegans* body muscle where it regulates both the sensitivity of muscle to ACh and the amount of ACh released from pre-synaptic neurons, via a retrograde messenger. miR-1 adjusts muscle sensitivity by directly regulating mRNAs encoding two subunits of the LevR (UNC-29 and UNC-63), whereas miR-1 adjusts pre-synaptic properties by regulating the mRNA encoding the transcription factor MEF-2. Acute activation of the LevR induces MEF-2-dependent transcription and the production of a MEF-2-dependent retrograde message. These results suggest that miR-1, LevRs, and MEF-2 define a nicotinic signaling pathway that couples post-synaptic activity with changes in pre-synaptic properties (Fig. 7R).

A new function for miR-1

Fly and mouse miR-1 knockouts suffer early lethality due to defects in muscle proliferation (Sokol and Ambros, 2005; Zhao et al., 2007) whereas *C. elegans mir-1* mutants are viable, and muscle develops normally. There are several potential explanations for this discrepancy. First,

worm muscle precursors do not undergo extensive proliferation prior to myogenic differentiation; consequently, the proliferative defects observed in mouse and fly would not be expected to occur in worm *mir-1* mutants. Second, muscle differentiation in the worm differs from other metazoans in several ways. Worm body muscle precursors do not undergo cell fusions, instead forming mature mononucleate muscle cells. Third, several genes that are critical for normal myogenic development in other organisms (e.g. MEF-2 and MyoD) play no apparent role in the worm (Chen et al., 1992; Dichoso et al., 2000). These differences may explain why muscle development occurs relatively normally in *mir-1* mutants.

In general, microRNAs have been described to regulate early developmental processes. Given that muscles develop normally in *mir-1* mutants, we were able to identify novel functions for miR-1 in the regulation of mature neuromuscular synapses. Based on our results, we speculate that some microRNAs (like miR-1) will regulate both early developmental processes as well as the mature function of cells after development is completed. Consistent with a post-developmental function elsewhere, miR-1 expression persists through adulthood in both fly and mouse (Nguyen and Frasch, 2006).

microRNA regulation of subunit diversity in heteromultimeric receptors

Transcriptional regulation is thought to underlie changes in subunit composition of heteromultimeric receptors. For example, in vertebrates, nAChRs undergo a developmentally programmed switch from the γ to ε subunits that is mediated by changes in transcription of these genes (Gu and Hall, 1988; Missias et al., 1996).

We propose that miR-1 provides an alternative post-transcriptional mechanism to regulate the subunit composition of nAChRs. In *mir-1* mutants, the abundance of targeted subunits (UNC-29 and UNC-63) was increased and this was accompanied by decreased muscle sensitivity to ACh. These changes in muscle sensitivity are mediated in part by altered composition of LevRs; however, other aspects of the post-synaptic phenotype (e.g. the change in endogenous EPSC amplitude and kinetics) are mediated by other miR-1 targets (e.g. MEF-2).

The precise mechanism by which LevR function is altered by miR-1 remains unclear. These effects could arise from changes in several aspects of receptor biogenesis and function, including association with accessory subunits, assembly in the ER, trafficking to the cell surface, or function of mature receptors on the muscle surface. In principle, any of these receptor properties could be modified by altered subunit composition.

Based on our results, we speculate that microRNAs may be utilized to regulate other heteromultimeric receptor complexes in an analogous manner. Subunit composition is known to regulate the signaling properties of many classes of receptors, e.g. growth factor receptors and cytokine receptors. Regulation by microRNAs could provide a novel mechanism to rapidly alter the composition and signaling properties of these receptors.

MEF-2 mediates the generation of a retrograde synaptic signal

Retrograde signaling has been proposed as a mechanism to adjust pre-synaptic release to match post-synaptic excitability during development and ongoing synaptic activity. This phenomenon has been extensively studied at the *Drosophila* NMJ where a variety of post-synaptic disruptions each produce a retrograde signal to increase neurotransmitter release (Davis and Bezprozvanny, 2001). In *C. elegans*, genetic data suggest the presence of a retrograde message at synapses, however thus far the nature of this message remains unknown (Doi and Iwasaki, 2002). We show here that MEF-2 activity in body muscles induces a retrograde signal that inhibits pre-synaptic release of ACh.

One puzzling aspect of our results is the phenotype of *mef-2* single mutants. Since *mef-2* mutations eliminate the retrograde signal, one might expect that *mef-2* mutants would have increased ACh release; however, we observed decreased ACh secretion in *mef-2* single mutants. Several mechanisms could explain this discrepancy. First, the decreased ACh release could be caused by MEF-2 functions unrelated to retrograde signaling. Second, MEF-2 and the retrograde signal may not be active under normal growth conditions. It seems likely that a homeostatic pathway would only be induced following periods of unusually high activity, and consequently may be inactive under normal growth conditions. Third, in the absence of an inducing signal, Mef2 actively represses expression of some target genes (Shalizi and Bonni, 2005; van der Linden et al., 2007). Thus, some MEF-2 targets will be de-repressed in *mef-2* mutants. Consequently, *mef-2* loss of function mutations may result in a weak induction of the retrograde signal. Consistent with this idea, we observed decreased ACh secretion and increased RAB-3 fluorescence in *mef-2* mutants, both of which suggest an increase in retrograde signaling. Further experiments are required to distinguish between these possibilities.

Mammalian Mef2 proteins were previously implicated in synapse formation. RNAi mediated knockdown of rat Mef2A increased the number of excitatory synapses formed onto cerebellar and hippocampal neurons (Flavell et al., 2006; Shalizi et al., 2006). These results were interpreted as Mef2-mediated regulation of either synapse formation or stability. Thus, in both worm and mammalian studies, post-synaptic Mef2 regulates aspects of synapse development or function.

Although the mechanisms leading to Mef2 dependent changes in synapse formation in rat neurons have not been defined, it seems possible that retrograde regulation of pre-synaptic function may be involved in this process. Synapse refinement during development often involves competition among synaptic inputs, whereby weaker inputs are eliminated (Katz and Shatz, 1996; Sanes and Lichtman, 2001). We speculate that Mef2 effects on synapse formation in hippocampal neurons may be mediated by retrograde inhibition of synaptic inputs, leading to their elimination.

RAB-3 as an effector of retrograde signaling

Although we have not identified the MEF-2 regulated retrograde messenger, our results suggest that RAB-3 is the downstream target of this activity. Pre-synaptic RAB-3 levels were increased when retrograde signaling was induced (i.e. in *mir-1* mutants or following levamisole treatment) and these effects were dependent on MEF-2 activity in muscles. Moreover, the ACh secretion defects caused by *mir-1* and *rab-3* mutations were strikingly similar, and additive defects were not observed in double mutants. Together these results strongly support the idea that the miR-1/MEF-2 regulated retrograde messenger acts via changes in pre-synaptic RAB-3 activity.

What is the mechanism underlying the secretion defects in *mir-1* mutants? Genetic studies in mice suggest that Rab3 proteins act at a late stage in the synaptic vesicle cycle, after docking and priming, to promote calcium-evoked fusion (Schluter et al., 2004). Our analysis of *mir-1* mutants is entirely consistent with this phenotype. We observed decreased stimulus evoked release, and a decreased rate of endogenous EPSCs, whereas there was no change in vesicle priming, nor in the number of primed vesicles available for release. Together these results suggest that vesicle release probability was reduced in *mir-1* mutants, as previously proposed for mice lacking Rab3 function (Schluter et al., 2004). Decreased vesicular release probability could be caused by decreased calcium influx, or by decreased calcium sensitivity of release. Given the strong conservation of RAB-3 and MEF-2 function, it seems likely that Rab3 may also be a pre-synaptic effector responsible for retrograde signaling by Mef2 in mammalian neurons, and perhaps for other retrograde messengers.

Regulation of retrograde signaling by muscle activity

Several results suggest that the LevR/MEF-2 retrograde signal is induced by muscle activity. For example, activation of muscle LevRs induces MEF-2 transcriptional activity and the MEF-2 mediated retrograde alteration of presynaptic RAB-3. By contrast, activation of muscle ACR-16 receptors with nicotine did not induce the retrograde alteration of RAB-3. Thus, LevRs are selectively coupled to MEF-2 function, providing a mechanism to couple muscle depolarization to the generation of a retrograde message.

What confers activity-dependence on the retrograde message? Dephosphorylation of Mef2 induces its transcriptional activity, and this dephosphorylation is often mediated by the calcium-activated phosphatase calcineurin (Flavell et al., 2006; Shalizi et al., 2006; Shalizi and Bonni, 2005). The calcium-dependence of MEF-2 activity could account for the activity dependence of the retrograde message. Consistent with this idea, LevRs isolated from worm extracts co-purify with the *C. elegans* ortholog of calcineurin, TAX-6 (Gottschalk et al., 2005). Roughly 50% of the current produced by muscle LevRs is mediated by calcium (Richmond and Jorgensen, 1999). We propose that association of calcineurin with LevRs provides a mechanism to selectively couple activation of LevRs to induction of MEF-2 transcriptional activity and retrograde signaling.

Feedback inhibition by homeostatic plasticity is thought to provide a mechanism to maintain consistent synaptic function in the face of fluctuations in activity (Davis and Bezprozvanny, 2001; Turrigiano and Nelson, 2004). We propose that LevR/MEF-2 retrograde signal identified here is an example of such a homeostatic pathway. In this scenario (Fig. 7R), during periods of increased activity, synaptic currents through the LevR would activate MEF-2, which in turn would initiate a retrograde message culminating in inactivation of RAB-3 and decreased ACh secretion. Thus, this pathway provides a mechanism to couple muscle depolarization to homeostatic inhibition of excitatory input.

We also show that miR-1 regulates multiple aspects of retrograde signaling. The subunit composition and function of LevRs, and the expression of MEF-2 are regulated by miR-1, all of which would regulate induction of the retrograde signal. Consistent with these regulatory effects, retrograde signaling was constitutively active in *mir-1* mutants. These results suggest that miR-1 regulates the gain of this homeostatic signal. When miR-1 activity is high, the threshold for inducing the retrograde signal would be shifted to higher levels of muscle activity, whereas the converse would be true when miR-1 activity is low. Further experiments are required to determine which specific physiological conditions, or when during development miR-1 levels or activity are altered. Our results suggest that miR-1 provides a potential mechanism to adjust the intensity of retrograde signaling.

Materials and Methods

Detailed descriptions of all methods are provided in the on-line Supplemental Information.

Strains were maintained at 20°C on lawns of OP50 (for imaging and behavior) or HB101 (for electrophysiology). A complete list of strains utilized is provided in the Supplemental Methods.

Western Blots and Immunofluorescence

Membranes were isolated from worm extracts by ultracentrifugation, solublized in sample buffer, and immunoblotted as described previously (Dreier et al., 2005). Custom polyclonal antibodies were raised against recombinant UNC-29, UNC-63, and UNC-38 GST-fusion proteins. Commercial anti-GFP antibody (Clontech) was utilized. For whole mount immunostaining, worms were fixed with Bouin's and processed as described previously (McEwen et al., 2006).

Plasmids

All expression vectors are based on the pPD49.26 backbone (A. Fire). Standard methods were utilized to construct all plasmids. Full details will be provided upon request. A 2.4kb *myo-3* promoter was used for expression in body muscles. nAChR and *mef-2* cDNAs were isolated by RT-PCR. For 3'UTR reporter constructs, a myristoylated GFP was cloned into pPD49.26 followed by 3'UTR sequences derived from *unc-29* (KP#1299), *unc-38* (KP#1355), or *mef-2* (KP#1394). pJK129 contains GFP fused to a mutagenized *unc-29* 3'UTR, in which the nucleotide sequences of the three miR-1 binding sites were randomized.

Drug treatment assays—Young adult worms were transferred to plates containing 200μM levamisole and assayed for paralysis as described previously (Nurrish et al., 1999). For imaging and real-time PCR experiments, late L4 worms were transferred to mock treatment plates or plates containing 200uM levamisole or 30mM nicotine. After one hour RNA was harvested or worms were immobilized for imaging.

Electrophysiology—Electrophysiology was done on dissected *C. elegans* as previously described (Richmond and Jorgensen, 1999). All recording conditions, data acquisition, and analysis were as described (McEwen et al., 2006). Data analysis was carried out in Igor Pro using custom written software. Stimulus artifacts for stimulus evoked responses were removed for clarity. The on-line supplement contains a detailed description of our conditions and analysis.

Microscopy—Quantitative wide field fluorescence microscopy was performed using custom software as described previously (Dittman and Kaplan, 2006).

Real-Time PCR

Total RNA was extracted from synchronized L4 worms and reverse transcribed (RetroScript, Ambion), according to the manufacturer's suggestions. Quantitative Real-Time PCR (qPCR) reactions were performed using SYBR Green Supermix (BioRad) using a BioRad iCycler IQ. Relative abundance of *frm-4* and *rpl-32* mRNAs was determined using gene specific primers (sequences provided upon request).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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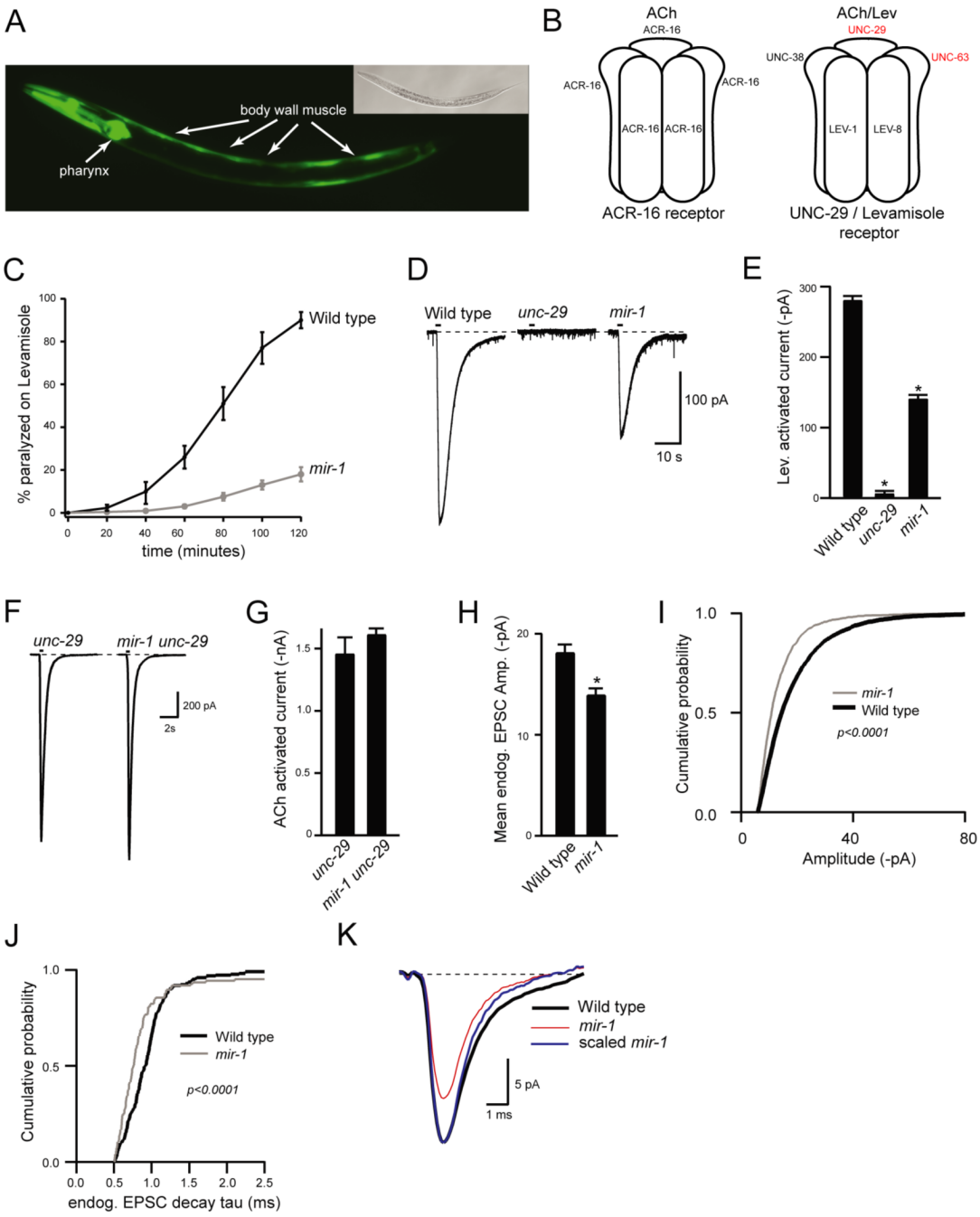

Figure 1.

miR-1 affects muscle sensitivity to ACh. A) A transcriptional reporter containing a 3.7kb *mir-1* promoter driving expression of GFP showed expression in body-wall and pharyngeal muscles. B) Body-wall muscle expresses two classes of nAChR: the ACR-16/α7 homo-pentamers and the levamisole sensitive hetero-pentamer containing UNC-29. The ACR-16 receptor is activated by ACh while the UNC-29 receptor is activated by both ACh and levamisole (Lev). C) The time course of levamisole (0.2 mM)-induced paralysis of Wild type and *mir-1(gk276)* was compared. D–E) Levamisole (100 μM, 0.5 s)-evoked currents in body muscles were compared in Wild type (n=18), *unc-29(x29)* (n=3), *mir-1(gk276)* (n=9). Averaged traces (D) and peak amplitudes (E) are shown. F–G) Acetylcholine (500 μM, 0.5 s)-

evoked currents in body muscles were compared in *unc-29* and *mir-1 unc-29* mutants. Averaged traces (F) and peak amplitudes (G) are shown. (*) indicates changes that are significantly different ($p<0.01$, Mann-Whitney) from control strains. Error bars indicate standard error of the mean. The mean endogenous EPSC amplitudes (H) and cumulative probability distributions of endogenous EPSC amplitudes (I) and decay taus (J) for *mir-1* and wild type controls are compared. K) Average endogenous EPSCs are shown for Wild type (black), *mir-1*(red) and a scaled version of *mir-1* (blue).

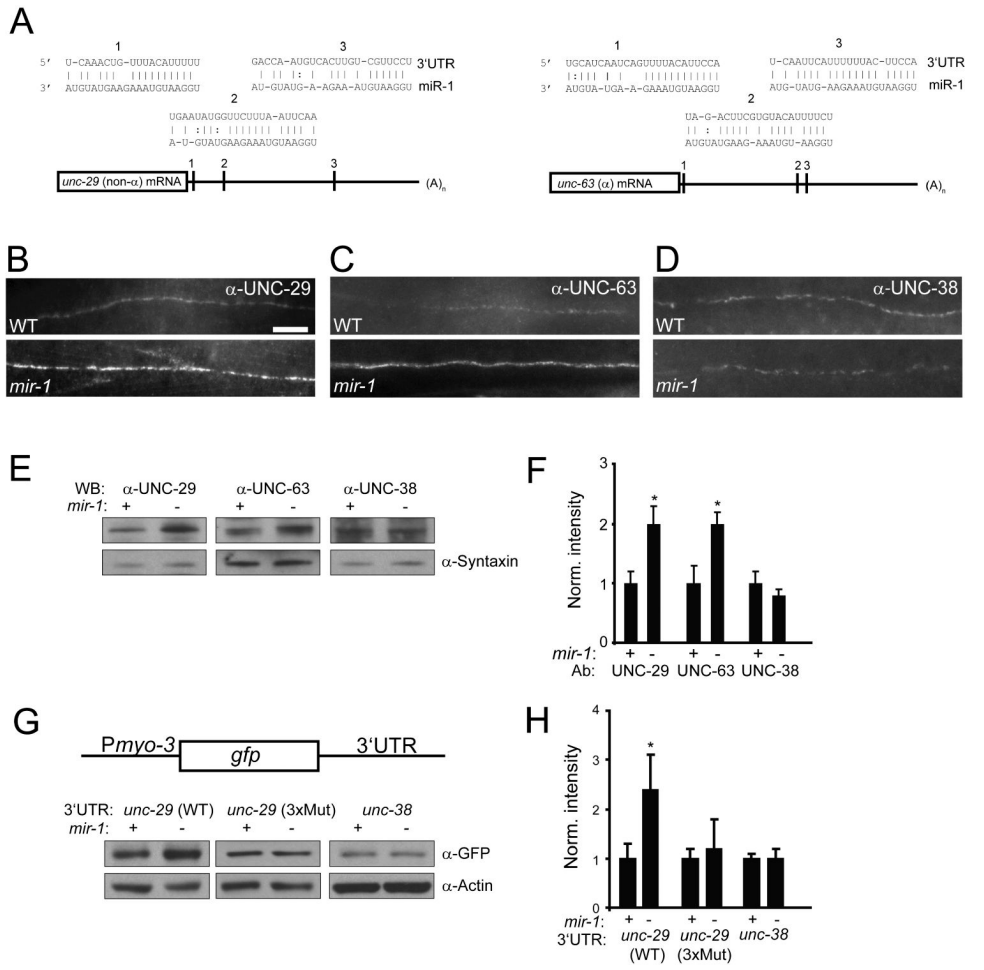

Figure 2.

miR-1 regulates nAChR subunit abundance. A) Sequence alignment of miR-1 binding sites (predicted using the Miranda algorithm) in the *unc-29* and *unc-63* 3'UTRs (Enright et al., 2003). (B–F) Abundance of endogenous UNC-29, UNC-38, and UNC-63 in wild-type (n=6) and *mir-1* mutants (n=6) was compared by immunofluorescence (B–D) and immunoblotting (E, F). Scale bar indicates 10 μm. G–H) GFP abundance in body muscles was measured in transgenic animals expressing constructs containing either a wild-type (WT) (n=6) or mutagenized (3xMut) *unc-29* 3'UTR (n=6), or the *unc-38* 3'UTR (n=6). In *unc-29* (3xMut), the sequence of the three miR-1 binding sites was scrambled (detailed in the methods). (*) indicates changes that are significantly different ($p<0.01$, Mann-Whitney) from control strains. Error bars indicate standard error of the mean.

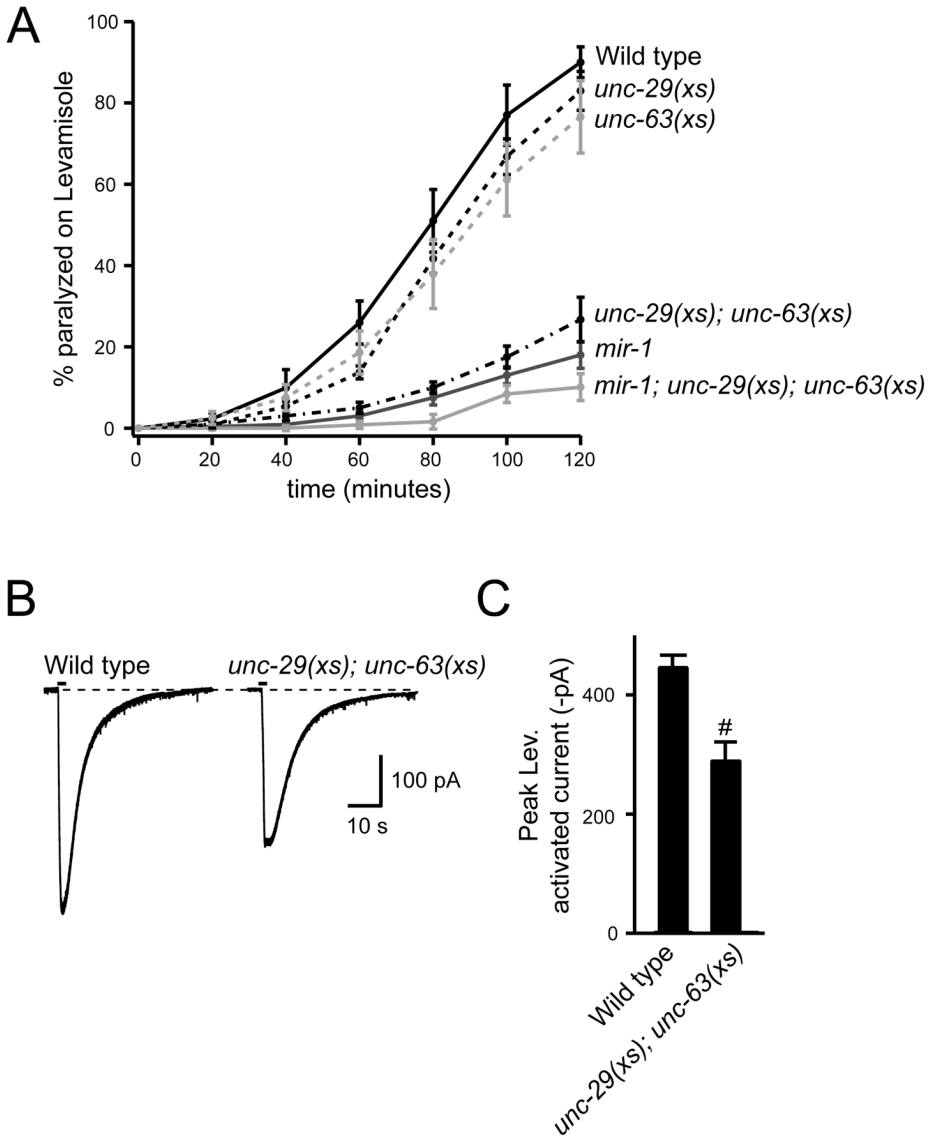

Figure 3.

Over-expression of UNC-29 and UNC-63 decreases sensitivity to levamisole. A) The time course of levamisole (0.2 mM)-induced paralysis was compared for wild-type, *mir-1(gk276)*, and transgenic animals over-expressing UNC-29 [*unc-29(xs)*], UNC-63 [*unc-63(xs)*], or both [*unc-29(xs)*;*unc-63(xs)*]. Data shown for Wild type and *mir-1(gk276)* are taken from Figure 1C. B–C) Levamisole (100 μM, 0.5 s)-evoked currents in body muscles were compared in wild-type (n=5) or transgenic animals over-expressing both *unc-29* and *unc-63* [*unc-29 (xs);unc-63(xs)*] (n=6). Averaged traces (B) and peak amplitudes (C) are shown. (#) indicates changes that are significantly different ($p<0.05$, Mann-Whitney) from control strains. Error bars indicate standard error of the mean.

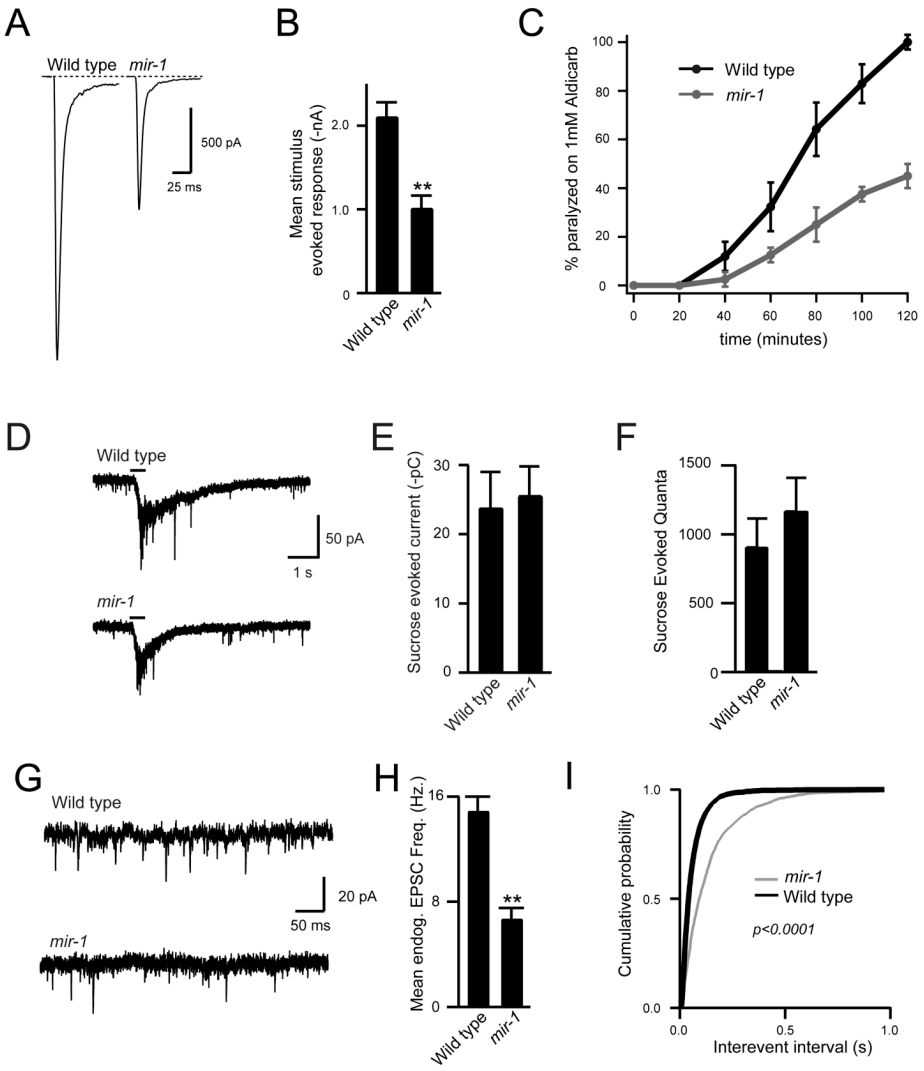

Figure 4.

Decreased pre-synaptic ACh secretion in *mir-1* mutants. Stimulus evoked responses were recorded from adult body wall muscles in 1mM $CaCl_2$, 4 mM $MgCl_2$. Average stimulus evoked responses (A) and EPSC amplitudes (B) are compared for Wild type (n=18) and *mir-1 (gk276)* (n=18) animals. For stimulus-evoked responses here and in subsequent figures, approximately 2 ms, encompassing the stimulus artifact, was blanked for clarity. Traces containing stimulus artifacts are presented in Supplementary Figure 9. C) The time course of aldicarb (1 mM)-induced paralysis was compared for wild-type and *mir-1(gk276)*. D–F) Sucrose evoked EPSCs were recorded from Wild type (n=6) and mir-1 (n=6) mutants. Representative sucrose responses (D), mean sucrose evoked charge transfer (1 second period after the stimulus) (E), and mean sucrose evoked quanta (F) are compared. Sucrose evoked quanta were computed by dividing the sucrose evoked charge transfer by the average endogenous EPSC charge transfer. G–I) Endogenous EPSCs were recorded from wild-type and *mir-1* adult animals. Representative traces (G), mean endogenous EPSC rates (H), and cumulative probability distributions for the interevent intervals (I) are shown. (**) indicates a significant difference ($p<0.0001$, Student's t-test) from Wild type.

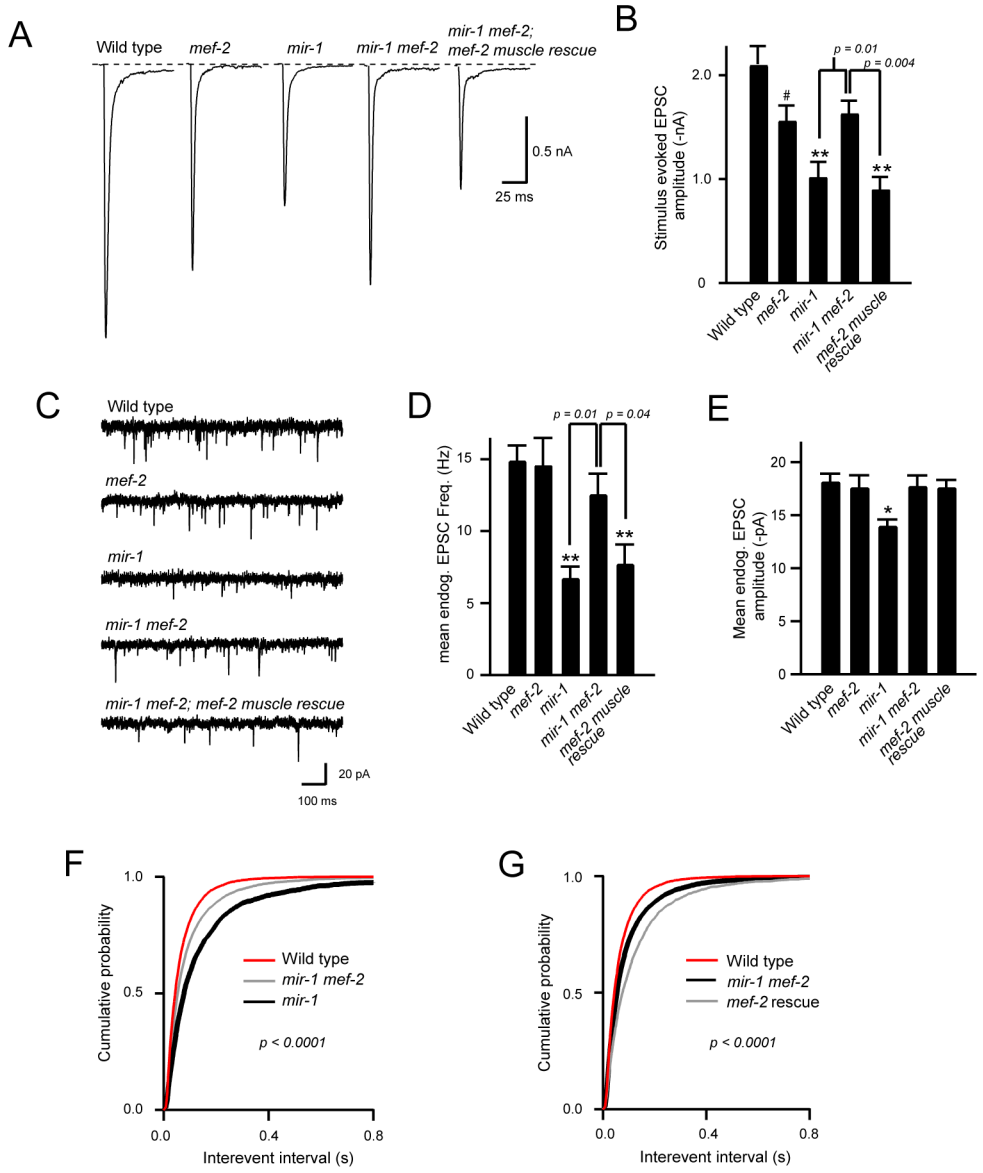

Figure 5.

MEF-2 mediates the pre-synaptic effects of miR-1. (A,B) Stimulus evoked responses were recorded from wild-type (n=18), *mef-2(gv1)* (n=8), *mir-1(gk276)* (n=18), *mir-1 mef-2* (n=9), and *mir-1 mef-2* double mutants carrying a transgene driving *mef-2* expression in body muscles (*mef-2* muscle rescue, n=6). Averaged responses (A) and mean EPSC amplitudes (B) are shown. C–G) Endogenous EPSCs were recorded from wild-type (n=18), *mef-2(gv1)* (n=9), *mir-1(gk276)* (n=18), *mir-1 mef-2* (n=9), and *mir-1 mef-2; mef-2* muscle rescue (n=6). The mean frequency (D), amplitude (E), and cumulative probability distributions of inter-event intervals (F,G) are shown. Values that differ significantly from wild-type controls are indicated: (*) $p<0.01$, (**) $p<0.001$, (#) $p<0.05$, Student's t-test. Error bars indicate standard error of the mean.

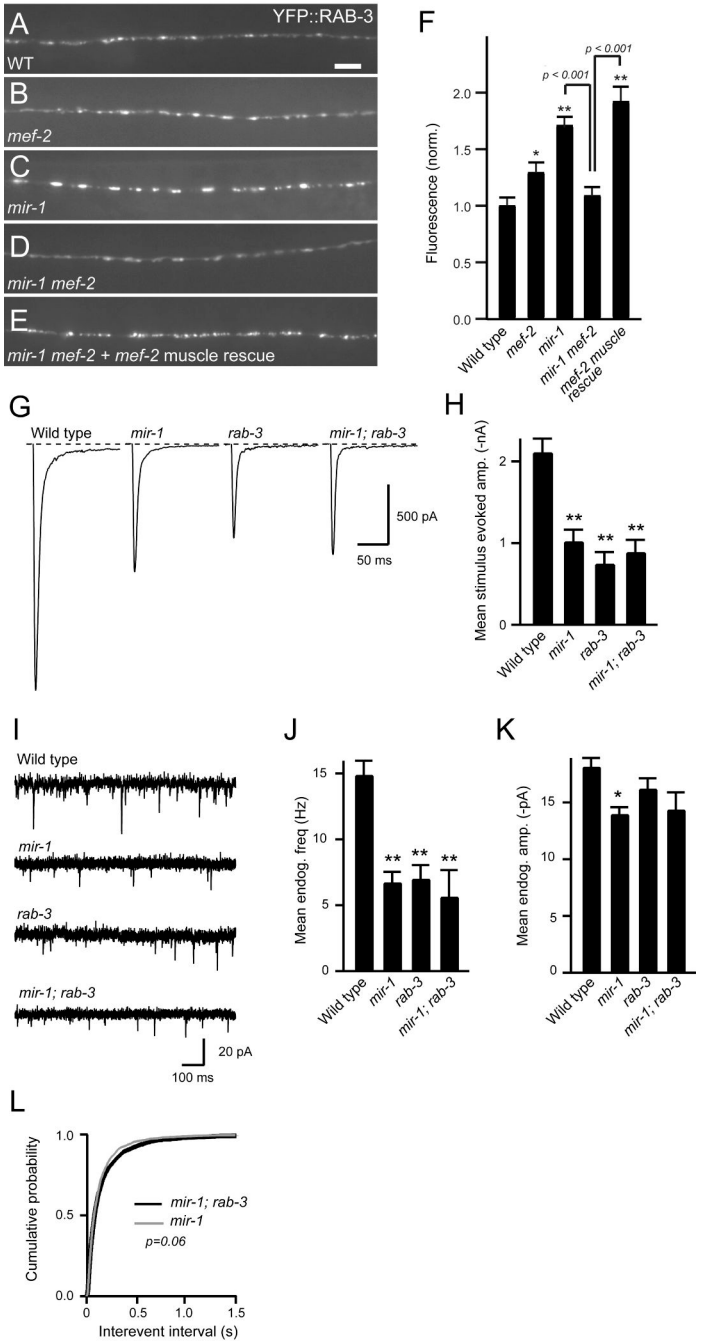

Figure 6.

RAB-3 is the pre-synaptic effector of the MEF-2-dependent retrograde message. A–E) YFP-tagged RAB-3 (YFP::RAB-3) was expressed in the cholinergic DA motor neurons (using the *unc-129* promoter) in the indicated genotypes. Scale bars indicate 10 μm. F) RAB-3 punctal fluorescence was compared in wild-type (n=50), *mef-2(gv1)* (n=65), *mir-1(gk276)* (n=51), *mir-1 mef-2* (n=34), and *mir-1 mef-2* double mutants with *mef-2* muscle rescue (n=29). Values that differ significantly from wild-type controls are indicated: (*) $p<0.01$, (**) $p<0.001$, Student's t-test. G–H) Stimulus evoked responses were recorded from Wild type (n=18), *mir-1 (gk276)* (n=18), *rab-3(js49)* (n=8) and *mir-1; rab-3* (n=6). Averaged responses (G) and EPSC amplitudes (H) are shown. I–L) Endogenous EPSCs were recorded from Wild type (n=18),

mir-1(gk276) (n=18), rab-3(js49) (n=8) and *mir-1; rab-3* (n=6). Representative traces (I), endogenous EPSC frequencies (J), and amplitudes (K), and cumulative probability distributions of inter-event intervals are shown for the indicated genotypes. Values that differ significantly from wild-type controls are indicated: (*) $p<0.01$, (**) $p<0.001$, Mann-Whitney.

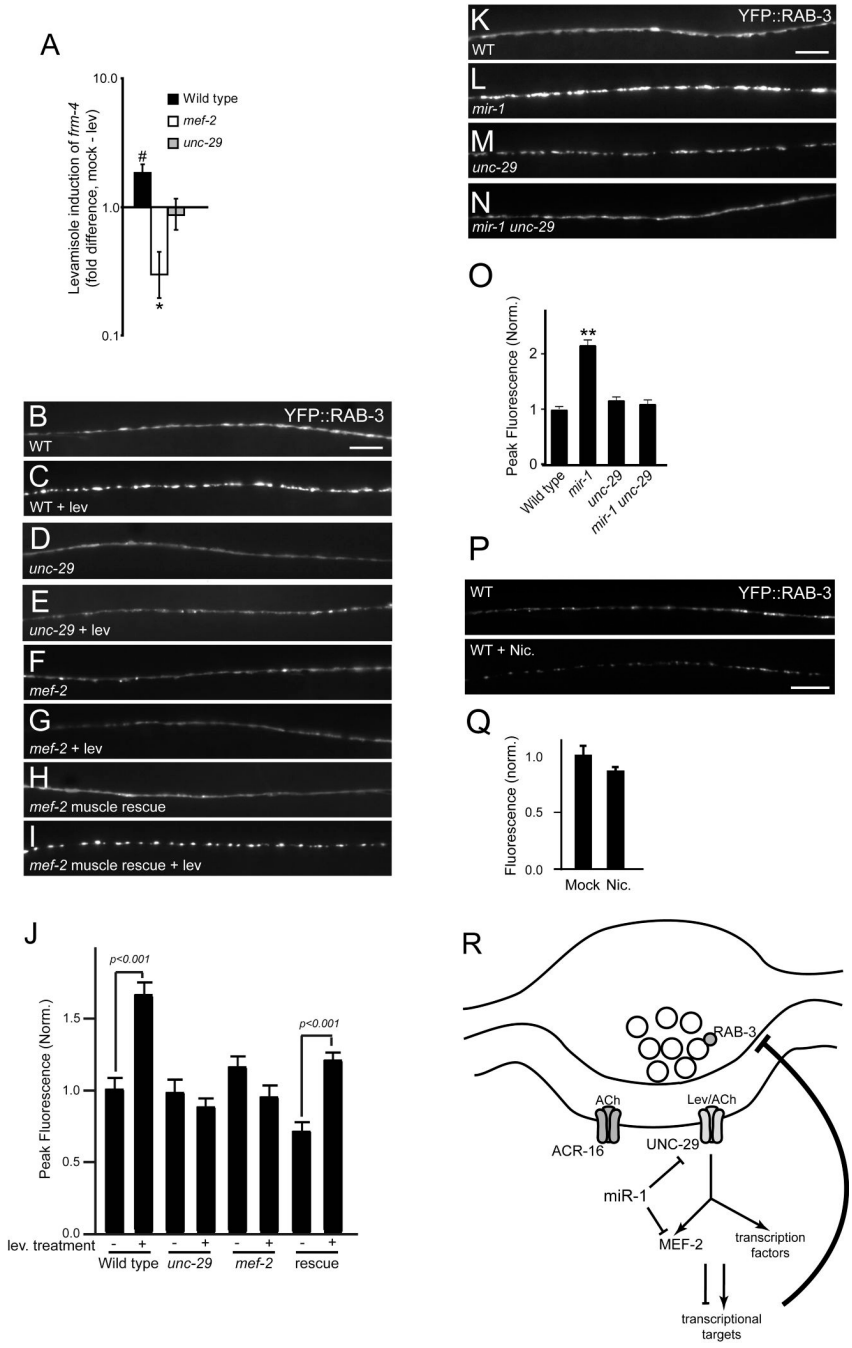

Figure 7.

Acute activation of LevRs initiates MEF-2-dependent changes in transcription and a retrograde change in YFP::RAB-3. After 1 hour, Levamisole (200μM) and mock treated animals were subjected to RNA extraction or imaging. A) Expression of *frm-4* was measured by qPCR (n=6 for Wild type and *mef-2*; n=3 for *unc-29*). Values that differ significantly from controls are indicated: (#) $p<0.05$, (*) $p<0.01$, Mann-Whitney. B–I) YFP::RAB-3 in the DA neurons is shown for the indicated genotypes. Scale bars indicate 10 μm. J) Average RAB-3 punctal fluorescence was compared in wild-type (n=25 mock, n=18 lev.), *unc-29(x29)* (n=16 mock, n=18 lev.), *mef-2(gv1)* (n=20 mock, n=16 lev.), and *mef-2* muscle rescue (n=12 mock, n=17 lev.). (K–O) *unc-29* mutations suppress the accumulation of YFP::RAB-3 in *mir-1* mutants.

YFP::RAB-3 punctal fluorescence is shown in the indicated genotypes. O) Average RAB-3 punctal fluorescence was compared in wild-type, *unc-29(x29)*, *mir-1(gk276)* and *mir-1 unc-29* double mutants [n=30 WT. n=26 *mir-1*, n=31 *unc-29*, n=35 *mir-1 unc-29*]. Values that differ significantly from wild-type controls are indicated: (**) $p<0.001$, Student's t-test. (P - Q) Nicotine treatment did not alter RAB-3 fluorescence in WT. Q) RAB-3 punctal fluorescence was compared in nicotine treated and control animals [n=33 mock, n=33 nic.]. R) A model for miR-1 regulation of the MEF-2 dependent retrograde signal. miR-1 regulates muscle sensitivity to ACh by regulating the LevR and the magnitude of pre-synaptic release by regulating the activity of MEF-2. We suggest that mis-regulation of MEF-2 either initiates or modulates a retrograde signal that inhibits ACh release, most likely by decreasing the activity of RAB-3. Our data are consistent with miR-1/UNC-29/MEF-2 acting as part of a nicotinic signal transduction pathway to couple muscle activity to the generation of a retrograde signal that inhibits neurotransmitter release.