The conserved microRNA miR-34 regulates synaptogenesis via coordination of distinct mechanisms in presynaptic and postsynaptic cells

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Micro(mi)RNA-based post-transcriptional regulatory mechanisms have been broadly implicated in the assembly and modulation of synaptic connections required to shape neural circuits, however, relatively few specific miRNAs have been identified that control synapse formation. Using a conditional transgenic toolkit for competitive inhibition of miRNA function in Drosophila, we performed an unbiased screen for novel regulators of synapse morphogenesis at the larval neuromuscular junction (NMJ). From a set of ten new validated regulators of NMJ growth, we discovered that miR-34 mutants display synaptic phenotypes and cell type-specific functions suggesting distinct downstream mechanisms in the presynaptic and postsynaptic compartments. A search for conserved downstream targets for miR-34 identified the junctional receptor CNTNAP4/Neurexin-IV (Nrx-IV) and the membrane cytoskeletal effector Adducin/Hu-li tai shao (Hts) as proteins whose synaptic expression is restricted by miR-34. Manipulation of miR-34, Nrx-IV or Hts-M function in motor neurons or muscle supports a model where presynaptic miR-34 inhibits Nrx-IV to influence active zone formation, whereas, postsynaptic miR-34 inhibits Hts to regulate the initiation of bouton formation from presynaptic terminals.
The molecular mechanisms controlling synapse formation, maturation, and stability have been studied in many contexts revealing extensive networks of signaling and structural proteins required on both sides of this specialized cellular junction. Understanding the regulatory logic that deploys and tunes these proteins to coordinate synapse morphogenesis simultaneously in both presynaptic and postsynaptic compartments remains an important research frontier.

Micro(mi)RNAs have emerged as ideal candidates to regulate nervous system development, plasticity and function. miRNAs can concurrently regulate the stability and translation of distinct target miRNAs via complementary miRNA response elements (MREs) that rely on conserved “seed” sequences to confer robust yet imprecise base-pairing with target transcripts. After initial discovery of these small non-coding RNAs and key downstream loci by genetic analysis, genomic and informatics technologies were developed to identify many hundreds of conserved miRNAs and predict their direct target miRNAs in many animal species. Subsequent miRNA profiling of neurons and synapses revealed that neurons express a particularly rich landscape of potential regulatory functions with significant potential for cell-type-specific mechanisms.

Driven largely by candidate approaches, a gradually increasing number of individual miRNAs have been found to shape and modulate neural networks. miRNA regulation of individual gene targets is known to control different aspects of synaptic morphogenesis, including dendritic spine modification, axonal sprouting and synaptogenesis.
miR-34 is required for multiple aspects of NMJ synapse Morphogenesis. One tremendous advantage of the Drosophila NMJ for studies of RNA regulatory mechanisms is the wealth of genetic data that define effector genes and pathways important for synapse development in this system\(^{38–42}\). To help prioritize miRNAs for deeper characterization, we compiled a database of genes with published larval NMJ phenotypes (Supplementary Data 2) and combined this with our list of Drosophila melanogaster (dme) genes orthologous to homo sapiens (hsa) genes with synaptic annotations. In parallel, we took our target predictions, and asked which of these fall within the combined synaptic set. We then ranked our ten novel miRNA candidates with these data as an estimate of enrichment for predicted synaptic target genes (Supplementary Fig. 1b). Several of our miRNA hits ranked highly, although some of these were members of large miRNA families (e.g. miR-92a/b or miR13a). The top-ranked candidate (miR-34) is a highly conserved, single gene miRNA (Supplementary Fig. 1b), whose mature form (dme-miR-34-5p) displays 87% sequence conservation to its three human homologues hsa-miR-34a-c-5p (Supplementary Fig. 1c). With this level of conservation in both the miRNA and possible synaptic target genes, the robust phenotype of miR-34 was an obvious choice for deeper characterization. In addition, although miR-34 family miRNAs had been implicated in different aspects of neural plasticity and development\(^{43–47}\), the precise functions of miR-34-family genes in presynaptic development had not been described.

Our analysis of gross L3 M6/7 NMJ architecture in miR-34 LOF animals showed that this miRNA is required to promote motor axon terminal arbor expansion and the formation of presynaptic boutons (Figs 1b and 2a-b); however, branching of the NMJ arbor was not dependent on miR-34 (Supplementary Fig. 2g). Moreover, observed decreases of type Ib bouton number at M12 NMJ was not dependent on miR-34 (Supplementary Fig. 2g). Moreover, observed decreases of type Ib bouton number at M12 NMJ was not dependent on miR-34 (Supplementary Fig. 2g). Moreover, observed decreases of type Ib bouton number at M12 NMJ was not dependent on miR-34 (Supplementary Fig. 2g). Moreover, observed decreases of type Ib bouton number at M12 NMJ was not dependent on miR-34 (Supplementary Fig. 2g). Moreover, observed decreases of type Ib bouton number at M12 NMJ was not dependent on miR-34 (Supplementary Fig. 2g).

miR-34 is required for normal postsynaptic cytomatrix (Fig. 2e), raising the possibility of altered organization on both sides of the synapse (Fig. 2d). More detailed analysis of type 1 boutons with specific markers for components that make up key presynaptic structures, such as the T-bars of active zones (AZs) visualized with anti-Bruchpilot (Brp), or the postsynaptic cytomatrix within the subsynaptic reticulum (SSR) visualized with Dlg, demonstrated defects in miR-34 mutants indicative of altered organization on both sides of the synapse (Fig. 2c, d). Significant increases in Brp intensity (Fig. 2c; Supplementary Fig. 2a–f) and Brp puncta number (Supplementary Fig. 2i) suggested elevated density or assembly of sites for presynaptic glutamate release, consistent with a gross increase in glutamate receptor staining (Supplementary Fig. 2b, e). However, the significant decrease in Dlg intensity (Fig. 2d) suggested a postsynaptic SSR defect. Analysis with an antibody recognizing a second SSR biomarker Syndapin\(^{18}\) confirmed that miR-34 is required for normal postsynaptic cytomatrix (Fig. 2e), raising the

**Fig. 1** In vivo screen of miRNA regulatory complexity in the Drosophila NMJ. a, c, d Scanning confocal images of NMJ arbors at larval muscles 6 and 7 (L3 segment A2) are shown stained for the presynaptic membrane (HRP, magenta), and postsynaptic cytomatrix (Dlg, green); scale bar (10 μm) applies to all panels. Representative phenotypes from the screen are shown, including a w\(^{1118}\) control, c miR-14 null undergrowth, and d miR-316 null overgrowth. b Bouton number phenotypes that reached or exceeded significance (p ≤ 0.05) are shown as a percentage change (Error bars indicate ± SEM) relative to matched control for GenII miR-SP driven ubiquitously using a tubulin-GAL4 driver (orange bars; tubulin-GAL4,Scramble-SP is the control genotype at 122.4 ± 11 SEM; sample depth of n = 10 hemisegments) or as confirmed by subsequent analysis of miRNA null alleles (blue bars; w\(^{1118}\) is the control genotype at 123.7 ± 8.75 SEM; sample depth of n = 10 hemisegments, except in the cases of miR-219 and miR-277 that were sampled and n = 20). All results shown were analyzed by ANOVA. Two tailed T-test and show p-values ≤ 0.05 (see Supplementary Data 1 for screen data). e Summary of bouton number defects observed in the miR-SP ubiquitous competitive inhibition assay, relative to novel phenotypes confirmed with null alleles (see key for categories). Source data are provided as a Source Data file.
Fig. 2 miR-34 is required for both presynaptic and postsynaptic development. a, b Scanning confocal images of the M6/7NMJ (segment A2) were stained for the presynaptic membrane (HRP, magenta), and postsynaptic (Dlg, green). a w^{1118} control animal; b miR-34 null. Scale bar (10 µm) applies to panels a and b. c–e Intensity quantification of synaptic biomarkers at the M6/7NMJ are shown for control and miR-34 null (** indicates p-value ≤ 0.01; ***p-value ≤ 0.001). c Presynaptic T-bar marker Brp imaged using 3-D SIM for high spatial resolution (n = 6 control and miR-34 NMJs). d, e Postsynaptic marker Dlg (n = 21 control and miR-34 NMJs) and Syndapin (n = 26 control and n = 23 miR-34 NMJs), respectively, imaged with confocal microscopy. f Quantification of SSR area from TEM images (* indicates p-value ≤ 0.05) (n = 12 boutons from 3 w^{1118} animals; n = 16 boutons from 7 miR-34 null animals). g, h TEM micrographs of type 1 boutons from the M6/7NMJ; Scale bar in g, 500 nm. g A w^{1118} control shows presynaptic vesicles are evenly distributed and the postsynaptic SSR is organized into distinct membrane folds. h A miR-34 null NMJ is shown for comparison. i Electrophysiological recordings from control and miR-34 null M6/7NMJs. Input resistance was not significantly different (p = 0.4178) between control (7.9 ± 0.4 MΩ; n = 7) and miR-34 null (8.2 ± 0.6 MΩ; n = 8) by Kruskal–Wallis test. j miR-34 null animals show slight, but significant elevation in evoked EJP amplitude compared to control. Error bars indicate ± SEM, (**p-value < 0.05). ( p-value ≤ 0.05). k miniature mEJP frequency was significantly higher in the miR-34 null (***p-value ≤ 0.001), but mEJP amplitude was not significantly (ns) altered in the mutant (l). Source data are provided as a Source Data file.
question of whether these alterations correspond to some change in SSR membrane architecture. Moreover, when we quantified a class of boutons that normally lack the SSR in L3 larvae (e.g. type II boutons at M12/13NMI)\textsuperscript{19}, we found no significant decrease in the numbers of these small boutons in miR-34 mutants (Supplementary Fig. 2i), suggesting a correlation between SSR structure and type I bouton addition. To assess postsynaptic structure at high resolution, we next examined miR-34 null animals at the ultrastructural level using transmission electron microscopy (TEM).

The ultrastructure of normal type Ib synaptic boutons has been extensively characterized\textsuperscript{20}. In addition to large mitochondria, the presynaptic nerve terminal contains many clear-core presynaptic vesicles concentrated near electron-dense AZs that include characteristic, central T-bar structures (Fig. 2g and Supplementary Fig. 2m). Type 1b boutons are surrounded by complex muscle membrane folds (SSR; Fig. 2g) that extend glutamate receptor-rich endfeet which may provide signal compartmentalization to sculpt synaptic responses similar to dendritic spine function\textsuperscript{51,52}. Analysis of miR-34 null mutant AZs, light level staining for the SV marker Synaptotagmin (Syt) showed normal intensity in the mutant boutons compared to controls (Supplementary Fig. 2k). No significant difference was found in presynaptic area (Supplementary Fig. 2f). However, compared to control (Supplementary Fig. 2m), the AZ ultrastructure of miR-34 nulls displayed abnormal and diffuse morphology (Supplementary Fig. 2n–p). These observations confirmed that miR-34 is required for both presynaptic and postsynaptic morphogenesis.

Although synaptic vesicles (SV) were often too dense to count by TEM proximal to miR-34 null mutant AZs, light level staining for the SV marker Synaptotagmin (Syt) showed normal intensity in the mutant boutons compared to controls (Supplementary Fig. 2k). No significant difference was found in presynaptic area (Supplementary Fig. 2f). However, compared to control (Supplementary Fig. 2m), the AZ ultrastructure of miR-34 nulls displayed abnormal and diffuse morphology (Supplementary Fig. 2n–p). These observations confirmed that miR-34 is required for both presynaptic and postsynaptic morphogenesis.

The impact of miR-34 loss on overall bouton number combined with an increase in AZ density and diminished SSR area raised the question of whether synaptic transmission might be altered in this mutant, or whether reduced NMJ arbor was sufficient to reduce NMJ growth to levels approaching the severity of ubiquitous expression of miR-34 SP (Fig. 3b, c, e (red bars); displayed relative to appropriate driver

miR-34 plays different roles on the two sides of the synapse. Although we observed significant structural phenotypes on both sides of the synapse in miR-34 null animals, it remained possible that these defects reflected a single underlying target gene mechanism acting in either motor neurons or muscle cells. To determine the cellular source of miR-34 action for NMJ growth, we used our miR-34SP under control of cell type-specific GAL4 drivers. Interestingly, we discovered that miR-34 is required on both sides of the synapse to achieve normal bouton numbers. Expression of miR-34SP specifically in the muscle compartment with DMeF2-GAL4 is sufficient to reduce NMJ growth to levels approaching the severity of ubiquitous expression of miR-34 SP (Fig. 3b, c, e (red bars); displayed relative to appropriate driver

![Figure 3](https://example.com/figure3.png)

**Fig. 3** Tissue-specific function of miR-34 for bouton addition is revealed by conditional inhibition. **a–d** Scanning confocal images of M6/7NMI (segment A2) stained for the presynaptic membrane (HRP, magenta), and postsynaptic (Dlg, green). (a) ubiquitous Scramble-SP expression with tubulin-GAL4 driver, (b) ubiquitous GenII miR-34SP expression with tubulin-GAL4 driver, (c) muscle-specific GenII miR-34SP expression with DMeF2-GAL4 driver, (d) motor neuron-specific GenII miR-34SP expression with OK6-GAL4. Scale bar (10 mm) applies to **a–d**. **e** Quantification of bouton number changes relative to Scramble-SP crossed with appropriate driver control for the GenII miR-34SP competitive inhibitor expressed under control of tubulin-GAL4 (n = 20 for ScrambleSP and miR-SP), DMeF2-GAL4 (n = 21 for ScrambleSP and n = 21 for miR-SP), or OK6-GAL4 (n = 20 for ScrambleSP and n = 21 for miR-SP), or the UAS-miR-34(+ ) overexpressor expressed under control of OK6-GAL4 (n = 20 for OE and control). Significant decrease in bouton number (red bars) or increase in bouton number (green bar) are shown. Error bars indicate ±SEM (***p-value ≤ 0.001). Source data are provided as a Source Data file.
crossed with Scramble-SP controls) or the null mutation (Fig. 1d). However, when we inhibited miR-34 presynaptically using a motor neuron-specific driver (OK6-GAL4) we observed the opposite phenotype resulting in NMJ overgrowth (Fig. 3d) with a significant increase in bouton number (Fig. 3e, green bar). Because miR-34 inhibition in motor neurons gave a surprising result, we asked if overexpression of miR-34 might yield a reciprocal effect. Indeed, expression of a UAS-miR-34+ strain under control of OK6-Gal4 caused a significant decrease in type 1 bouton number relative to the OK6-Gal4 control (Fig. 3e, far right red bar); unfortunately, expression of UAS-miR-34+ in muscle was early lethal. These results indicate that miR-34 plays different roles in the presynaptic and postsynaptic compartments, suggesting a working hypothesis that the key downstream target genes in motor neurons and muscle cells are distinct.

A search for conserved miR-34 targets identifies synaptic effector proteins. To help elucidate the contrasting synaptic roles of miR-34 in motor neurons and muscles, we set out to identify target genes relevant to NMJ morphogenesis. MRE search algorithms could predict a large number of candidate targets (376), however, because MREs alone are insufficient to predict biological target activity [reviewed in Ref. 54], we expected many candidates to be irrelevant at the synapse. Thus, we next defined the overlap of potential miR-34 targets with our database of synaptic effector genes including known Drosophila NMJ phenotypes (Supplementary Data 2) plus orthologs of genes annotated in SynaptomeDB55 that could be identified using Dipt. This overlap identified 56 candidate synaptic genes. However, MRE sequence prediction alone is not consistently correlated to functional miRNA-dependent translational repression54. Moreover, rapid evolution of these short regulatory sequences is thought to produce many species-specific target genes55. Therefore, we asked how many of the 56 predicted miR-34 synaptic targets displayed significant sequence conservation between Drosophila and human for both the encoded structural gene and the miR-34 seed sequence complement. This final criterion yielded only three conserved proteins with documented synaptic functions that were likely to be regulated by miR-34 from edcsyoza to vertebrata: Adducin/Hu-li tai shao [Hts56–59, CNTNAP4/Neurexin-IV|Nrx-IV60,61 and LRRCT7(Densin-180)l/Lap1. Although LRRCT7/Densin-180/Lap1 has yet to be studied at the Drosophila NMJ, Nrx-IV and Hts are known to control aspects of NMJ development that overlap with the phenotypes we discovered in miR-34 mutants. In addition to the glial functions of Nrx-IV to promote cell–cell and junctional interactions60,62,63, this conserved cell surface molecule also regulates the assembly of AZs and bouton addition in presynaptic motor nerve terminals. Indeed, elevation of Nrx-IV is sufficient to significantly increase bouton addition and Brp accumulation60, thus phenocopying presynaptic effects of miR-34 inhibition. No muscle functions for Nrx-IV have been described; however, the cytoskeletal regulator Hts has been shown to play roles in both motor neurons and muscle cells at the larval NMJ. Presynaptic function of Hts controls exploratory membrane protrusion and synapse elimination, presumably by regulating actin cytoskeleton56. In muscle, Hts has been shown to regulate the assembly of the postsynaptic cytomatrix57,64, including SSR localization of the scaffolding protein Dlg that we find to be dependent on miR-34. These data thus lead us to a working model that the predicted and conserved miR-34 targets Nrx-IV and Hts might account for distinct aspects of the miR-34 synaptic phenotype.

Nrx-IV can account for the presynaptic function of miR-34. Under normal conditions, the junctional adhesion receptor Nrx-IV is highly expressed in glial cells that ensheathe motor axons as they enter the target area65,66 (Supplementary Fig. 3a, b). Interestingly, Nrx-IV protein also accumulates in motor neuron terminals, where it clusters adjacent to presynaptic T-bar structures60. Using confocal microscopy and 3-D SIM to image presynaptic boutons beyond the glial footprint, we confirmed Nrx-IV localization in puncta that surround the Brp-positive center of each AZ (Fig. 4a). However, when we compared miR-34 mutant and control NMJs under identical image acquisition conditions, we discovered a consistent and significant increase in the size and intensity of endogenous Nrx-IV puncta (Fig. 4b; quantitative comparison in Fig. 4c from 3-D SIM data); this was consistent with the presence of a conserved miR-34 MRE in the Nrx-IV 3’ untranslated region (UTR; Fig. 4d). In contrast, Nrx-IV staining intensity along the main axon arbor where glial cells reside did not show a significant increase in miR-34 mutants (Supplementary Fig. 3c).

To test the prediction that elevation of Nrx-IV selectively in motor neurons might be sufficient to phenocopy the NMJ growth phenotype of miR-34, we used the OK6-Gal4 driver to over-express Nrx-IV (under the control of GAL4 UAS). As predicted, elevation of Nrx-IV in motor neurons induced a significant increase in type 1b bouton number that was qualitatively comparable to motor neuron-specific inhibition of miR-34, albeit quantitatively milder when normalized to the GAL4 control (Fig. 4e). To show that Nrx-IV is required for the NMJ overgrowth induced by presynaptic inhibition of miR-34, we then performed a rescue experiment where Nrx-IV was reduced by RNAi in motor neurons that also express miR-34SP. When compared to scrambleSP controls driven by the same OK6-GAL4, reduction of Nrx-IV effectively rescued presynaptic growth in the miR-34SP background (Fig. 4f), thus supporting a model where miR-34 limits presynaptic growth by attenuating expression of Nrx-IV. To be thorough, we also performed a control to determine if a second, inert UAS transgene (UAS-GFP-L10a) might simply reduce the efficiency of the UAS-miR-34BP by diluting GAL4 activity; however, the phenotype of miR-34SP was not significantly altered by the second UAS site (Supplementary Fig. 3d). Interestingly, OK6-GAL4:UAS-Nrx-IVRNAi alone caused significant increase in bouton number (Supplementary Fig. 3e). Although regulation of Nrx-IV expression could account for presynaptic miR-34 phenotypes, it remained formally possible that Nrx-IV might exert control of NMJ growth on both sides of the synapse. Thus, we used musclespecific DMef2-GAL4 to drive postsynaptic overexpression of Nrx-IV; however, no significant change in bouton number was observed (Fig. 4e, gray bar), further supporting the model that miR-34-dependent restriction of Nrx-IV expression is required selectively in motor neurons.

Postsynaptic regulation of hts accounts for miR-34 control of bouton addition. The Drosophila Hts gene produces at least four protein isoforms through differential RNA splicing. Although multiple Hts isoforms are likely to be expressed in L3 body wall muscle64, the major isoform(s) thought to accumulate in the postsynaptic SSR contain the C-terminal MARCKS domain that binds to F-actin and is recognized the antibody Hts-M56,57,67 (see Supplementary Fig. 4a for a diagram of the Hts transcription unit and Hts-M antigen). We confirmed this corona-like localization surrounding large boutons using anti-Hts-M in combination with presynaptic HRP (Fig. 5a, a’). However, when we compared Hts-M intensity in miR-34 mutants versus genetically matched controls using identical confocal imaging conditions (Fig. 5a, a’ versus b, b’), we found a consistent and significant elevation of the Hts-M antigen by at least 1.5-fold within the synaptic...
compartment (quantified in Fig. 5c). There was also a marked increase in Hts-M expression throughout the muscle fibers (compare Fig. 5a, b, and a” to b”). These data suggested that synaptic Hts-M isoform levels are negatively regulated by miR-34, consistent with the presence of a conserved miR-34 MRE in the 3′ UTR downstream of the MARCKS domain-encoding exon (Fig. 5d; Supplementary Fig. 4a).

To test whether selective elevation of the Hts-M isoform(s) might be sufficient to mimic the NMJ defects that we observed in either miR-34 null mutants or miR-34SP competitive inhibition, we used a wild type UAS-hts-Mwt cDNA transgene lacking the miR-34 3′ MRE for cell type-specific overexpression of Hts-M. Using a Dmef2-GAL4 driver, we found that a single transgene insertion (UAS-hts-MwtVK33) displayed an intermediate increase

Fig. 4 Restriction of synaptic Nrx-IV protein can account for motor neuron inhibition of miR-34. a, b Presynaptic active zones marked by the T-bar constituent Brp (green) are surrounded by small punctate accumulations of Nrx-IV protein (magenta) in the terminal boutons of the M6/7NMJ (segment A2; Scale bar: 5 μm). b In miR-34 null animals, an identical staining and imaging protocol reveals a consistent and significant increase in the size and intensity of Nrx-IV puncta compared to control in a. c The difference in intensity between synaptic Nrx-IV signals is quantified (n = 6 NMJs per genotype; *p-value ≤ 0.05). d Alignment of the conserved MRE for Human (hsa) miR-34a within the 3′UTR of CNTNAP4 is shown in comparison to the corresponding MRE for Drosophila (dme) miR-34 in the Neurexin-IV (Nrx-IV) mRNA. e Motor neuron-specific competitive inhibition of miR-34 (n = 21) using OK6-GAL4 to express miR-34SP is compared to Nrx-IV overexpression (Nrx-IV OE) of under OK6-GAL4 (green bar; n = 19) or a muscle-specific DMef2-GAL4 (gray bar; n = 20) (**p-value ≤ 0.01; ***p-value ≤ 0.001) all relative controls n = 20. f OK6-GAL4 expression of miR-34SP induces a significant increase in type 1 bouton numbers per NMJ (green bar; n = 20) compared to Scramble-SP controls (white bar; n = 21); this phenotype is completely rescued by co-expression of UAS-Nrx-IVRNAi (gray bar; n = 20) (***p-value ≤ 0.001). Source data are provided as a Source Data file.
in Hts-M expression compared to control or a 2x UAS-hts-M insert (Supplementary Fig. 4d). When we counted mature type I boutons in the single copy UAS-hts-MwtVK33 combined with DMef2-GAL4, we discovered that bouton number decreased at the muscle 6/7 NMJ by nearly 30%, producing a phenotype that was indistinguishable from that observed in DMef2-GAL4;miR-34SP (Fig. 5e); this phenotype was also highly reminiscent of the effect on the muscle 4 NMJ when UAS-hts-M was expressed with DMef2-GAL4 in a previous study. To further verify our finding that Hts-M overexpression in muscle is sufficient to restrict type I bouton addition, we used DMef2-GAL4 to drive expression of a single insertion cDNA transgene from a separate source (UAS-htsS704S from Wang and colleagues) in comparison to the matched GAL4 control. We observed a significant albeit milder reduction in type I bouton number (Supplementary Fig. 4e). Consistent with previous pan-neuronal overexpression of...
Fig. 5 Regulation of Hts-M isoforms in muscle accounts for miR-34 promotion of bouton addition. a, b MARCKS domain-containing Hts-M protein isoforms (green) surround presynaptic boutons stained with anti-HRP (magenta) at the M6/7NMJ. Hts-M signal is observed in the muscle with high levels surrounding type 1 boutons in w1118 control (a) and miR-34 null (b) NMJs (segment A2; Scale bar: 10 μm). High magnification insets separating the HRP (a’, b’) and Hts-M (a”, b”) channels (merged in a”, b”) reveal increased Hts-M signal and beyond the SSR halo surrounding large boutons in miR-34 mutants. c Fluorescence intensity profiling of Hts-M antigen within a region of interest surrounding the NMJ and adjacent muscle (see Methods) reveals a significant increase in immunolabeling (‘p’ ≤ 0.05). d Alignment of the conserved MRE for Human (hsa) miR-34a within the 3’ UTR of Adducin2 (Add2) is shown in comparison to the corresponding MRE for Drosophila (dme) miR-34 in the Hts 3’ UTR downstream of the MARCKS domain coding sequence. e Muscle-specific competitive inhibition of miR-34 using DMef2-GAL4 to express miR-34SP compared to Hts-M isoform overexpression (Hts-M OE via UAS-hsts-MVKK32) using the same DMef2-GAL4 driver (left and right red bars), reveals highly significant deficits in type 1 boutons that are not significantly different from the decrease observed in miR-34SP (p = 0.79). However, motor neuron-specific OK6-GAL4 driven Hts-M OE fails to induce a significant change in bouton number (gray bar); n = 20 NMJs for each genotype including control lines which differences are graphed relative to. f DMef2-GAL4 expression of miR-34SP induces a significant decrease in type 1 bouton numbers per NMJ (red bar) compared to Scramble-3P controls (white bar); this phenotype is completely rescued by co-expression of UAS-hstsRNAi (gray bar) n = 20 NMJs for each genotype. g Using Mhc-GAL4 and the same UAS-hstsRNAi transgene, we also observe effective rescue of the miR-34 null type 1 bouton phenotype; n = 20 for Control, n = 19 for miR-34 Null, n = 20 for Muscle Rescue. h Parallel experiments using spaced depolarization to induce activity-dependent nascent boutons show that controls (w1118 above, Df-Mef2-GAL4 below) show highly significant increases after K+ treatment compared to mock-treated samples (green bars), whereas, both miR-34 null (above) and UAS-hsts-MVKK32 over-expression (OE, below) produce no induction (gray bars). For c and e-h Error bars indicate ±SEM; “∗”p-value ≤ 0.05; “∗∗”p-value ≤ 0.01; “∗∗∗”p-value ≤ 0.001; ns is p > 0.05; for h upper panel: w1118 and miR-34 mock n = 13 for, K+ + n = 9, w1118 mock n = 8, K+ + n = 11; for h lower panel: DMef2-GAL4 experiments Hts-M OE mock n = 24, K+ treated n = 18, control mock n = 13, K+ treated n = 11. i A working model of miR-34 action in the presynaptic (green) and postsynaptic compartments of type 1 boutons showing how regulation of Nrx-IV in motor neurons can influence active zones while regulation of Hts-M isoforms controls formation of nascent boutons, possibly via modulation of remodeling of SSR and/or synaptic adhesion. Source data are provided as a Source Data file.
neuronal differentiation in vertebrates43,45. miR-34 MREs are conserved in the CNTNAP4 and Adducin gene families from insect to human, suggesting that these regulatory mechanisms are ancient. In both vertebrate and invertebrate nervous systems, CNTNAP-family receptors are well known to mediate glial-neuronal and glial-glial cell surface interactions65,66,70,71. However, recent observations at the Drosophila NMJ showed that Nrx-IV can modulate AZ assembly and synapse growth60,65 much like the related receptor Nrx-I70. These studies and our findings predict that precise tuning of Nrx-IV levels is essential for normal synaptogenesis, consistent with our finding that miR-34 loss induces comparable presynaptic AZ defects (Fig. 2c and Supplementary Fig. 2a–f, i) as elevation of Nrx-IV60,65. Precise levels of CNTNAP receptors also appear to be vital during human brain development, as dosage changes in CNTNAP4 and CNTNAP2 are associated with autism spectrum disorders60,70,72.

In addition to altered AZ structure, presynaptic-specific inhibition of miR-34 or elevation of Nrx-IV results in an increased addition of boutons in the motor neuron terminal arbor (Figs. 3c and 4e)65, suggesting mechanistic coordination between AZ assembly and synapse growth, as has been observed for the peri-AZ scaffolding protein Liprin-alpha and the associated receptor tyrosine phosphatase LAR70,73. However, with respect to bouton formation during larval development, the postsynaptic role of miR-34 reveals a muscle imposed limitation on nascent bouton formation, presumably by regulating cytoskeletal structure and cell-cell interaction. This raises the intriguing possibility that mechanisms promoting presynaptic growth must be coordinated with concomitant postsynaptic remodeling, presumably involving one or more trans-synaptic signaling systems. It also remains possible that miR-34 controls additional targets yet to be identified that may contribute to shaping synaptic development, function and/or plasticity. In future, we hope to expand our search for such effectors to better understand the underlying mechanisms and how miR-34 functions in concert with other miRNAs uncovered by our screen.

Previous studies have shown that the MARCKS domain of certain Adducin/Hts protein isofoms mediates a physical bridge between the barbed end of F-actin and the membrane cortex-associated spectrin-Ankyrin network, thereby conferring stability of cellular structure56,74–78. In neurons, this mechanism provides a powerful means for calcium-dependent kinase regulation of the Adducin MARCKS domain to drive presynaptic structural plasticity and formation of memory in response to patterns of neural activity56,58, but relatively little is known about the postsynaptic regulation upstream of Adducin-like Hts-M isofoms. In this regard it is intriguing that miR-34 is required for activity-dependent bouton growth (Fig. 5h), hinting that the miR-34/Hts-M mechanism may be activity-regulated in some way. In addition to a dependence on miR-34 (Fig. 2d–f), SSR architecture has been shown to remodel in response to neuronal activity79. Although it is not clear whether SSR at the Drosophila NMJ is truly analogous to the dendritic spines of mammalian glutamatergic synapses, as has been suggested80, mammalian miR-34 family members do control the formation of dendrites and dendritic spines43. This raises the possibility that miR-34 regulation of Adducin may play a conserved role in postsynaptic development and/or plasticity.

A tight correlation between bouton addition and SSR structure is also observed in mutants lacking the miRNA miR-819. Analogous to miR-34 regulation of Hts-M, miR-8 promotes bouton addition in larval development by muscle-specific repression of the actin assembly factor Enabled (Ena) that localizes within the SSR18,19. Together, analysis of miR-8 and miR-34 suggest that plasticity or dynamics in postsynaptic architecture may be essential for the formation or maturation of new presynaptic varicosities in this system. In addition to cytoskeletal structure, postsynaptic Hts has been shown to influence the localization of the cytomatrix scaffold Dlg that is required to build the SSR77. Interestingly, elevation of all Hts transcripts in muscle77 has been observed to cause a different effect on NMJ structure than selective elevation of Hts-M (Fig. 5e and Supplementary Fig. 4e), highlighting the question of whether the ratio or local regulation of different isoforms might play an important role in regulating bouton initiation. Because Hts has also been shown to have an impact on additional downstream factors during synapse plasticity, such as CamKKII and Par-184, the underlying mechanism may also involve a cascade of signaling events. In adult neurons and muscles, miR-34 is expressed and required to protect cells from age-dependent degeneration57,58,59,81–83. Given Adducin’s role in controlling structural stability, it may be interesting to ask if this highly conserved miR-34 target influences the course of neurodegeneration in aging animals.

In conclusion, our analysis of miR-34 revealed novel regulatory relationships required to shape features of the synapse that depend on cellular interaction between presynaptic and postsynaptic partners. The fact that miR-34 phenotypes reflect complex regulation of disulphide bridge generation, storage and muscle highlights a strength of genetic models where condition expression or inhibition can be used to illuminate simultaneous contributions of different neighboring cells. Interestingly, like miR-34, the majority of miRNA that regulate NMJ morphogenesis appear to promote bouton addition, including eight of the ten novel miRNA validated with nulls in our study (Fig. 1) and three of four miRNAs previously known to control NMJ growth (miR-124, miR-310–313; miR-8; miR-1000/137)18,84–86. This suggests that synapse growth control is tuned by restricting expression of many factors that negatively regulate bouton addition. In the case of Adducin/Hts-M, negative regulation may be achieved by promoting stability or by the need for coordinated cytoskeletal remodeling of the postsynaptic cytomatrix and presynaptic boutons. However, the identity and cellular specificity of target genes downstream of the other novel miRNAs remain to be determined.

Methods

**Drosophila strains and genetics.** All stocks were maintained at 25°C according to standard procedures. The miR-SP and Scramble-SP lines were generated by the Van Vactor laboratory; in addition to 131 GenII miR-SP lines (attP-40attP-2) used for the primary screen that are now available at the Bloomington Drosophila Stock Center (BDSC, Bloomington, IN, USA)66, a third generation (Gen III) set of miR-SPs was made with identical SP insertion sequences using a pUASt/attB vector with DsRed2 (cloned by Genewiz) and distinct insertion sites (attP-ZH538;attP-VK33; Bestgene strain number 24482 and 9750, respectively) to control for genetic background, and to correct for gradual observed loss of GenII miR-SP penetrance over many generations with the 20xSP array in attP-40attP-2 sites since the original primary screens66; these new lines are available upon request from the Van Vactor laboratory until deposited at the BDSC. Most of the miRNA nulls used in this study were generated by and obtained from the Cohen laboratory26, miR-34 null was a kind gift from the Bonini lab83. miR-277 null was a kind gift from Chun-Hong Chen. A miR-316 null indel was generated by CRISPR (Supplementary Fig. 5). The UAS-miR34 OE line was obtained from Norbert Perrimon and colleagues. The UAS-miR34 OE insertion Hts overexpression line, was obtained from the Pielage lab68. UAS-Hts(20746) was obtained from the Kreiger lab64, w118, w725, UAS insertion at Nrx-IV BL17985, Nrx-IV RNAi BL28715 and GAL4 lines (Tab-GALA, OK6-GALA and DMe2(GAL4), UAS-GFP-L10a and UAS-GFP were obtained from BDSC. The UAS-hsRosa26 V29102 was obtained from the Vienna Drosophila Resource Center. For all experiments, we used control genotypes that were as closely matched to the background of the experimental strains as possible; for this reason, we have displayed data in percentage change from control in cases where the genotypes compared required distinct controls that may differ from each other.

**Bioinformatics.** Ranking of conserved miRNAs was based on percentage of predicted target genes identified in the mammalian SynapromeDB86. For our analysis, the following id mapping was completed using the indicated databases: microRNAs from the NMJ screen were mapped to a current miRBase id and accession number. Accessions were filtered with only mature miRNAs included. Mapping data between mirbase accessions and mirbase ids was completed using miRBase version
Fluorescent signal intensity was quantified using ImageJ to determine Raw Integrated Density (RawIntDen = sum of all values in selection for each channel) divided by the 2D area of interest (ROI). To define the presynaptic ROI for quantification of Dlg and Synapdin, a synaptic mask was generated with the ImageJ thresholding tool that captured the presynaptic terminal and the surrounding SSR area by using default settings based on the sum projection of the 3-D volume rendered for the HRP signal, followed by manual selection of the synaptic ROI to generate the ROIs. The ROIs were then applied to the sum projections for the test channel (e.g., Dlg or Synapdin). The final data output of RawIntDen and ROI Area were then generated using the ImageJ Analyze Particles tool. For Hts-M, because a significant amount of Hts-M protein accumulated outside of the HRP-visualized synaptic ROI, we generated a new synaptic ROI that included the entire synaptic arbor, SSR and adjacent muscle field; to avoid bias based on overall NMJ arbor size, we normalized for ROI area. Note that HRP intensity values did not vary significantly between control and miR-34 mutant NMJs. For statistical comparisons, a Student’s t-test was employed.

Structured illumination super-resolution microscopy. 3D-SIM data were collected on a DeltaVision OMX V4 Blaze system (GE Healthcare) equipped with a 1.42 N.A. Plan Apo oil immersion objective lens (Olympus), 488, 561, and 642 nm diode lasers, and a separate Edge 5.5 sCMOS camera (PCO) for each channel. Z-stacks of were acquired with a z-step of 125 nm and with 15 raw images per plane (five phases, three axes); the axial x-y pixel dimension is 40 nm. Spherical aberration was minimized using immersion oil matching wavelengths. Super-resolved images were computed from the raw image stacks of Z-planes with a channel-specific measured optical transfer function (OTF) and a Wiener filter constant of 0.001–0.002 using softWoRx 6.1.3 (GE Healthcare). Channel mis-registration was measured using a control slide and multi-channel datasets were registered using the image registration function in softWoRx. Channel images were then deconvolved by the measured channel OTF to reconstruct images from the original raw data. The images were calibrated using the ImageJ Analyze Particles tool. For volume measurements of Brp puncta, we used the 3-D Objects Counter tool in ImageJ on OTF 3-D rendered images after converting images to 16 bit and using the Auto Threshold tool.

Transmission electron microscopy. Larval third instar insects were dissected as described above and internal organs were removed. Following rapid dissection in Ca2+-free 0.1 M Coca-codylate buffer, the body walls were left pinned and fixed at 4°C overnight in 2.5% paraformaldehyde, 5% glutaraldehyde (vol/vol) and 0.06% picric acid (vol/vol) in 0.1 M Coca-codylate for 2 h on ice and then rinsed three times for 10 min in deionized water and dehydrated in an ethanol series (50, 70, 95, 100%, and 100% again, vol/vol) and post-fixed in 1% OsO4 and placed overnight in 1% aqueous osmium tetroxide (Taar) solution. The tissues were then stained with uranyl acetate and imaged on JEOL 1200EX 80-kV electron microscope at 60,000x and 25,000x magnification.

Electrophysiology. All larval dissections for electrophysiology were conducted as described in Inmlach and McBride, using third instar larvae in Hemolymph-Like saline solution 3.1 (HL 3.1) at a pH of 7.1–7.4, containing (in mM) 70 NaCl, 5 KCl, 4 MgCl2, 10 NaHCO3, 5 Trehalose, 115 Sucrose, and 5 HEPES (300 Osm). Body wall muscles were pinned to positions to optimize electrode placement and recording efficacy at neuromuscular junction (NMJ). EJPs were recorded intra-cellularly from muscle of abdominal segments 3–5 at 20–23°C in low Ca2+ (0.8 mM CaCl2 in HL 3.1 at a pH of 7.1–7.4), immediately following mEPSP recordings. A suction pipette with a tip opening of approximately 10 μm was used to stimulate the motor nerve (stimulation intensity was 0.1 ms, strength 5, 5 V, 10 Hz). Baseline membrane voltage was recorded for one second prior to stimulation; total stimulation lasted one second; three seconds of responses were recorded. Recordings were discarded if baseline varied by more than 5 mV during stimulation. All EJP recordings were analyzed in MATLAB. Frequency and amplitudes were measured via scripts and functions provided by Ted Brockers, Peter Bronk, and Stephen Alkmins. Minimum amplitude for mEJP = 0.4 mV.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.
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Author contributions
E.M.M. and D.V. conceived and supervised project; E.M.M., C.W., A.T., H.H. performed image and analysis of NMJ phenotypes and expression patterns; S.A. and L.C.G. performed EJP recordings; T.A.F. and E.M.M. created the miR-SP collection; T.F.D. and D.P.W. assisted in bioinformatics analysis; E.M.M. and D.V. wrote manuscript, with editorial suggestions from L.C.G.

Competing interests
The authors declare no competing interests.

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