miR-153 Regulates SNAP-25, Synaptic Transmission, and Neuronal Development

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Abstract
SNAP-25 is a core component of the trimeric SNARE complex mediating vesicle exocytosis during membrane addition for neuronal growth, neuropeptide/growth factor secretion, and neurotransmitter release during synaptic transmission. Here, we report a novel microRNA mechanism of SNAP-25 regulation controlling motor neuron development, neurosecretion, synaptic activity, and movement in zebrafish. Loss of miR-153 causes overexpression of SNAP-25 and consequent hyperactive movement in early zebrafish embryos. Conversely, overexpression of miR-153 causes SNAP-25 down regulation resulting in near complete paralysis, mimicking the effects of treatment with Botulinum neurotoxin. miR-153-dependent changes in synaptic activity at the neuromuscular junction are consistent with the observed movement defects. Underlying the movement defects, perturbation of miR-153 function causes dramatic developmental changes in motor neuron patterning and branching. Together, our results indicate that precise control of SNAP-25 expression by miR-153 is critically important for proper neuronal patterning as well as neurotransmission.

Introduction
Trimeric soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes form the core machinery mediating vesicular exocytosis [1–3]. In the nervous system, SNARE complexes are involved in membrane addition during neuronal growth as well as both dense core vesicle (DCV) release of proteins and synaptic vesicle (SV) release of fast neurotransmitters. At synapses, the core SNARE protein SNAP-25 interacts with accessory proteins that together regulate SV exocytosis by linking Ca²⁺ sensing to membrane fusion and neurotransmitter release [4–7]. SNAP-25 is a specific target of Botulinum neurotoxin that blocks vesicle release, resulting in rapid paralysis and death [8,9]. Misregulation of SNAP-25 is associated with several human diseases and neurodegenerative disorders including Huntington’s Disease [10], Alzheimer’s Disease [11], and diabetes [12].

SNAP-25 is required for action potential-evoked glutamatergic, cholinergic, and glycineergic transmission in neurons [13,14]. Mouse knockouts of SNAP-25 are therefore lethal although neuronal cultures from SNAP-25 null mutants maintain the ability to exhibit stimulus-independent transmitter release [13,15]. GABAergic inhibitory synapses express lower levels of SNAP-25 and may be more sensitive to calcium regulation, whereas glutamatergic excitatory synapses express higher amounts of SNAP-25 that alters calcium sensitivity [4]. Part of this differential regulation could be due to accessory proteins that control SNAP-25 distribution and levels to modulate synaptic activity [16–18]. Transcriptional mechanisms regulating SNAP-25 levels have also been suggested to play key roles in the dynamic control of synaptic function [19–23].

Several miRNAs have been shown to regulate synapse formation or homeostasis, mostly within the post-synaptic dendrite [22,24,25]. On the presynaptic side, most forms of regulation center on modulation of calcium channels and calcium-dependent vesicle release [26,27]. In this study, we show that miR-153 inhibits SNAP-25 expression in the developing nervous system. Precise control of SNAP-25 by miR-153 is necessary not only for presynaptic vesicle release, but also for protein secretion, motor neuron patterning, and outgrowth.

Results
miR-153 Regulates Embryonic Movement
miR-153 has been proposed to be one of a limited number of ancient miRNAs that evolved with the establishment of tissue identity [28]. It is conserved among bilaterians displaying distinct expression patterns in neurosecretory brain cells of the deuterostome marine worm Platynereis dumerilii and the protostome annelid Capitella [28]. In zebrafish, miR-153 is expressed in distinct regions of the developing nervous system and brain, including neurose-
miR-153 Targets snap-25

To identify mRNAs regulated by miR-153, we used target prediction algorithms, compared the expression patterns of both potential mRNA targets and miR-153, and assayed phenotypes from gain and loss of function experiments. Based on these criteria, snap-25 proved to be a bona fide target for miR-153 based on the results of reporter silencing experiments (Fig. 2) and consistent with conservation of miRNA recognition elements (MREs) from fish to humans (Fig. S2).

There are two SNAP-25 paralogs in zebrafish (a and b isoforms) with similar, but not identical, 3′ UTRs [33,34]. For reporter assays, we fused the 3′ UTR from both snap-25 isoforms to the GFP reading frame (snap-25a data shown in Fig. 2A; snap-25b shown in Fig. S3). Synthetic miRNAs prepared from these reporters were injected into single cell embryos in the presence or absence of exogenous miR-153 or miR-153 morpholinos (MOs). Based on fluorescence levels in live embryos at 1 dpf, co-injection of miR-153 resulted in obvious down-regulation of GFP for both isoforms (Fig. 2B). To confirm that the loss of GFP was due to pairing with the predicted MREs, we created deletions of individual and combinations of MREs in snap-25a and snap-25b. Deletion of both MREs from snap-25a and all three MREs from snap-25b abolished the ability of miR-153 to silence expression (Fig. 2B; Fig. S3B). For snap-25a, we tested each of the individual MREs and found that deletion of a single MRE resulted in only modest silencing whereas deletion of both MREs caused a loss of silencing. We conclude that miR-153 targets both isoforms of snap-25 in an MRE-dependent manner.

If miR-153 targets snap-25, knockdown of endogenous miR-153 should lead to increased reporter fluorescence. To test this prediction, antisense morpholinos were co-injected with reporter mRNAs (Fig. 2). We found that knockdown of miR-153 caused a significant increase in GFP expression compared to embryos with wild type levels of endogenous miR-153. Lastly, we performed western blots using antibodies against GFP and analyzed protein...
levels were determined by western blotting using antibodies against GFP or control antibodies against snap-25a,b overexpression of mRNA with mRNAs (Fig. 1; Movie S1). Knockdown of snap-25a,b predicted miRNA recognition elements (MREs) were identified in the snap-25a,b UTR sequence is shown in green. (B) Single cell zebrafish embryos were injected with miRNAs derived from GFP reporters lacking a UTR (GFP), fused to the full length snap-25a UTR (+snap-25a), or mutant versions of the snap-25a UTR lacking individual MREs (snap-25aΔMRE1 and snap-25aΔMRE2) or both MREs (snap-25aΔMRE1&2). Embryos were injected in the presence or absence of exogenous miR-153 or morpholinos against miR-153 (miR-153MO). Fluorescence levels were examined at 1 dpf. Clusters of embryos (~60) are shown as well as a high magnification image of a single representative embryo. (C) Lysates from ~100 embryos were prepared from embryos treated as in B and GFP protein levels were determined by western blotting using antibodies against GFP or control antibodies against α-tubulin. (D) Quantitation of westerns was performed with a paired Student’s t-test (n = 5).

SNAP-25 is a known target of Botulinum neurotoxin (BoNT) proteases A and E [8,9]. If miR-153 is targeting snap-25a, the effects of increased miR-153 should mimic the effects of BoNT A. To test this prediction, injected zebrafish were exposed to BoNT A for 30 minutes at 27 hpf. One hour later, western blots were performed on pooled protein samples to determine whether it was possible to rescue SNAP-25 over-expression phenotypes associated with miR-153 knockdown or injection of snap-25a,b mRNAs. Exposure to BoNT A dramatically reduced SNAP-25 levels, recapitulating the effects of miR-153 knockdown and over-expression (Fig. 4A,B). For movement, exposure to BoNT A rescued the hyperactive phenotype observed after injection with MOs against miR-153 or overexpression of snap-25a,b mRNAs (Fig. 4C; Movie S1). Together, these experiments strongly support the conclusion that miR-153 specifically targets snap-25 to regulate embryonic movement.

miR-153 Regulation of Motor Neuron Development

SNAP-25 is a well-characterized t-SNARE protein, with an established function in vesicular exocytosis [1–3]. In the developing nervous system, the SNARE complex mediates vesicular membrane addition driving neurite outgrowth and morphological patterning [1–3,35]. Moreover, DCV-mediated release of signaling proteins and growth factors is important for axon guidance, path finding, and morphological development [36–39]. We therefore sought to determine whether snap-25 regulation by miR-153 would alter neuronal morphogenesis. Because zebrafish motor neuron development is well characterized [40–45], we decided to focus on the effects of miR-153 on motor neurons during early zebrafish development.

We first injected miR-153 or morpholinos against miR-153 to observe the effects on the development and morphology of motor neurons in a transgenic zebrafish line in which motor neurons are specifically labeled with RFP (Tg(nxn1:1TagRFP-T) [46]. Perturbation of miR-153 levels caused striking changes in motor neuron structure and branching (Fig. 5A,B). Compared with NICs, overexpression of miR-153 dramatically changed the axonal architecture with significant decreases in branch numbers and length (Fig. 5C, D). Knockdown of miR-153 resulted in completely opposite effects with increased motor projection architectural complexity, increased axonal length, and increased branch numbers (Fig. 5B-D). To test whether the effects were specific, we conducted rescue experiments, as above. Injection of snap-25a,b mRNA or morpholinos against snap-25a/b produced virtually the same phenotypes observed in embryos subjected to miR-153 knockdown or overexpression, respectively. In contrast, co-injection of miR-153 and snap-25a,b mRNAs or morpholinos against miR-153 and snap-25a,b almost completely restored the normal pattering and branching of motor neurons (Fig. 5B-D). These results indicate that miR-153 regulates motor neuron development via control of snap-25a,b.

To further dissect the function of miR-153 on motor neuron development, immunofluorescence was performed on whole-mount zebrafish embryos (55 hpf) with antibodies that label...
primary (Znp-1 or anti-synaptotagmin 2) or secondary (Zn-8 or Alcama) motor neurons [47]. Compared to NIC embryos, a striking difference in primary motor neuron axon architecture was observed with both miR-153 overexpression (miR-153) and knockdown (miR-153MO) (Fig. 6). A significant decrease in branching was observed in miR-153 injected embryos whereas knockdown of miR-153 caused a dramatic increase in branching. Likewise, injection of snap-25a,b mRNA led to increased axonal growth and branching in primary motor neurons whereas knockdown of snap-25a,b caused decreased outgrowth and branching (Fig. 6). Co-injection experiments showed that snap-25a,b mRNA and morpholinos against snap-25 could partially counteract the effects of the corresponding gain and loss of miR-153.

For secondary motor neurons, rostral axon outgrowth was similarly stunted and/or irregularly spaced by miR-153 overexpression and slightly elongated by miR-153 knockdown (miR-153MO) (Fig. 6). A significant decrease in branching was observed in miR-153 injected embryos whereas knockdown of miR-153 caused a dramatic increase in branching. Likewise, injection of snap-25a,b mRNA led to increased axonal growth and branching in primary motor neurons whereas knockdown of snap-25a,b caused decreased outgrowth and branching (Fig. 6). Co-injection experiments showed that snap-25a,b mRNA and morpholinos against snap-25 could partially counteract the effects of the corresponding gain and loss of miR-153.

Expression of miR-153 in Motor Neurons

To ensure that the effects of miR-153 on motor neuron patterning were due to expression of miR-153 in these cells, we FACS sorted cells from the trunks of 52 hpf (Tg(mnx1:TagRFP-T)) embryos and conducted RT/qPCR. As shown in Fig. 7, there was a greater than 10-fold enrichment for miR-153 in RFP+ cells compared to RFP- cells. Prior work had shown that miR-153 is expressed in the brain and spinal cord but these results show that miR-153 is expressed in developing motor neurons.
Since SNAP-25 has a well-established function in the fusion and release of numerous vesicle types, we next examined the role that miR-153 plays in modulating exocytosis. Owing to the core role of miR-153 in movement control, we first focused on synaptic activity at the neuromuscular junction (NMJ) in zebrafish embryos. For this analysis, we measured synaptic vesicle (SV) cycling using the styryl dye, FM1-43 [48,49]. At 55 hpf, embryonic NMJs were imaged with Alexa 594-conjugated α-bungarotoxin (α-Btx) to label postsynaptic acetylcholine receptor (AChR) clusters, while monitoring FM1-43 uptake into NMJ presynaptic boutons (Fig. 8). The terminals were acutely depolarized for 5 minutes with high [K+] saline (45 mM) to drive the SV cycle and load FM1-43, whereas only weak loading was evident in low [K+] conditions. In non-injected controls, fluorescence was observed along terminal axon branches with intense staining at individual synaptic varicosity boutons (Fig. 8A). Compared to NIC labeling, miR-153 over-expression resulted in a significant decrease in FM1-43 loading in presynaptic terminals, indicating slowing of the SV cycle (Fig. 8B). In sharp contrast, knockdown of miR-153 showed a significant increase in FM1-43 loading, indicating an elevated SV cycling rate (Fig. 8C). The significant difference between miR-153 knockdown and overexpression conditions indicates that miR-153 plays an important role in controlling the rate of vesicle cycling (Fig. 8D). Together, these results reveal a key function for miR-153 in the control of presynaptic vesicle release at the embryonic NMJ, consistent with a role for miR-153 in the regulation of embryonic movement. The overall effects on movement are therefore a combination of effects on motor neuron development and patterning as well as overall exocytic activity.

SNAP-25 has a highly conserved role mediating vesicular fusion in both neurons and other neurosecretory cells where it is critical for DCV release [50]. To test whether miR-153 plays a role in this secretory context, we examined exocytosis in a rat neuroendocrine pituitary cell line (GH4C1) expressing human growth hormone (hGH) [51]. Release of hGH in these cells provided a functional readout of exocytic activity (Fig. 9). GH4C1 cells were therefore transfected with miR-153, morpholinos against miR-153/snap-25, or vectors expressing snap-25a,b, followed by determination of hGH levels in the media by ELISA. Overexpression of miR-153 and knockdown of snap-25a,b (snap-25abMO) reduced the levels of hGH to below the amount detected in culture media from mock transfected cells (Fig. 9). In sharp contrast, knockdown of miR-153 and overexpression of snap-25 both significantly increased the amount of secreted hGH 8–10 fold over the mock transfected control (Fig. 9). The differences observed due to perturbation of miR-153 levels in the GH4C1 cell line compared to embryonic NMJs are most likely due to differences in the efficiency of miR-153/miR-153MO delivery between the two experiments, as well as developmental differences. Nevertheless, the effects in this case were fully suppressed by co-expression of either miR-153/snap-25a,b mRNA or MOs against miR-153/snap-25a,b, demonstrating specific regulation of snap-25 by miR-153. These data strongly support the conclusion that miR-153 functions to precisely control SNAP-25 levels to regulate vesicle exocytosis.

**Discussion**

In this study, we show that miR-153 regulates the critical core SNARE component, SNAP-25, to modulate exocytosis and neuronal development. Increased miR-153 levels cause decreased SNAP-25 expression resulting in decreased embryonic movement, decreased neuronal secretion, and decreased neuronal growth/...
branching. Conversely, miR-153 knockdown causes elevated SNAP-25 expression resulting in hyperactive movement, increased neuronal secretion, and increased neuronal growth/branching. Accumulating evidence suggests that SNAP-25 misregulation plays a role in numerous human disease states including ADHD, schizophrenia, bipolar I disorder, Huntington’s disease, Alzheimer’s disease, and diabetes [52]. Regulated expression of miR-153 provides an attractive model to mechanistically explain tight control of SNAP-25 levels.

SNAP-25 Functions during Development

It is well established that axon outgrowth during neuronal development occurs via SNARE-dependent addition of membrane for growth cone extension [35,53]. Axonal growth, pathfinding, and target recognition are secondarily modulated by SNARE-dependent release of developmental signals via dense core vesicle (DCV) exocytosis [54–59]. The outgrowth of both axons and dendrites is blocked by Botulinum neurotoxins A and C1, proteases specific for SNAP-25, demonstrating a direct role of SNAP-25 in neuronal morphogenesis [55,56,60]. Likewise, in-
Inhibition of SNAP-25 by antisense oligonucleotides blocks axonal outgrowth [54]. In stark contrast, neuronal outgrowth was surprisingly not inhibited in SNAP-25 null mice [13]. The explanation for this inconsistency is not clear. Our results show a clear requirement for SNAP-25 in motor neuron outgrowth and branching in zebrafish. It is possible that the requirement for SNAP-25 may be species specific but we found that altered levels of miR-153 caused similar branching defects in rat PC12 cells as observed in zebrafish motor neurons, strongly arguing against this (data not shown). Perhaps the differences are due to cell-specific requirements for SNAP-25. In the retina, for example, SNAP-25 is expressed in a dynamic spatiotemporal pattern and such differential expression may underlie specific development of cholinergic amacrine cells and photoreceptors [61]. An intriguing possibility based on the results presented here is that developmental, stage-specific and/or cell-specific expression of miR-153

**Figure 6.** miR-153 regulates primary motor neuron development. (A) Immunofluorescence performed on whole mount zebrafish embryos at 55 hpf using Znp-1 antibodies to label primary motor neurons. Confocal images were acquired from the same somites for all embryos, as indicated. (B) Effects on primary motor neuron structure and branching under the indicated conditions. Scale bar: 40 μm.
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SNAP-25 in Synaptic Vesicle Exocytosis

SNAP-25 is one of three SNARE proteins that contribute α-helices that mediate fusion between synaptic vesicles and pre-synaptic membranes [1,3]. Blockage of synaptic transmission by Clostridium and Botulinum neurotoxins first established that SNARE proteins are critical for neurotransmitter release [62]. Cleavage of SNAP-25 by Botulinum neurotoxin A causes a paralytic phenotype that resembles the loss of movement we observe in zebrafish embryos expressing excess miR-153. SNAP-25 haploinsufficient mice show no observable phenotypic defects but complete loss of SNAP-25 blocks evoked synaptic transmission [13]. Moreover, overexpression of SNAP-25 inhibits normal calcium responsiveness and can impair memory-associated synaptic plasticity [63]. These findings suggest that modulation of SNAP-25 levels are important for overall SNARE function, especially in generating differences in calcium dependence between neuronal and non-neuronal secretory vesicular fusion events. Matteoli and colleagues (2009) have shown that SNAP-25 is differentially expressed between excitatory glutamatergic and inhibitory GABAergic neurons in a developmental-specific manner [4]. These results remain controversial, as earlier studies did not observe this difference, but the data are consistent with an important role for SNAP-25 as a required component for both glutamatergic and GABAergic transmission [64,65]. Mechanisms for how SNAP-25 levels might be regulated in a development- and/or cell-specific manner are uncertain, but our data strongly support miRNA regulation as a likely candidate and a critical mechanism controlling SNAP-25 levels. A recent report describing the effects of chronic overexpression of SNAP-25 in the rat dorsal hippocampus demonstrated the critical importance of controlling SNAP-25 levels [63]. Elevated expression of SNAP-25 produced increased levels of secreted glutamate with cognitive deficits similar to those observed in ADHD and schizophrenia. We propose that miR-153 control of SNAP-25 levels allows for precise regulation of SNAP-25 during development and exocytosis.

miRNAs Regulation of Neuronal Morphogenesis and Synaptic Activity

Localized translation control in synaptic dendrites is common, requiring repression of mRNA translation during transport. miRNA mediated inhibition of translation is an attractive mechanism that can precisely control gene expression in neurons. Consistent with this hypothesis, many miRNAs are neuron or brain specific [66]. Moreover, the effector complexes that carry out repression of translation (RNA Induced Silencing Complexes; RISCs) are composed of several subunits that have been implicated in both neuronal function and disease [22,24,67]. For example, nervous system specific miRNAs have been shown to regulate the maturation of dopamine neurons in the midbrain as well as control serotonin transport by regulating the serotonin transporter [68,69]. Likewise, miR-1, miR-124, miR-125b, miR-132, bantam, miR-34 and the miR-319 cluster have all been implicated in the modulation of synaptic homeostasis [70–76]. Similarly, synaptic plasticity is reportedly regulated by miR-134 through targeting of SIRT1 or Limk1, which control dendritic spine morphogenesis [77,78]. In addition, miR-124 in retinal ganglion cell growth cone was shown to act through CoREST to regulate the intrinsic temporal sensitivity to Sema3A, a guide cue during axonal pathfinding and morphogenesis [79]. Our work demonstrates that miR-153 is a member of this subset of miRNAs implicated in neuronal function but by a distinctly different mechanism through targeting of snap-25. miR-153 also likely targets other miRNAs [80], but SNAP-25 regulation alone is required and sufficient to explain the role of miR-153 regulation of movement, motor neuron morphogenesis, and SNARE-mediated secretion.

Materials and Methods

Ethics Statement

The Animal Care and Use Committee monitors all animal care and research at Vanderbilt. Vanderbilt University Institutional Animal Care and Use Committee (IACUC) under protocol M-09-398. In accordance with that protocol, all necessary means were taken to avoid pain. For any manipulations that might induce pain, animals were anesthetized with a 0.15% solution of Tricaine (3-amino-benzoic acid ethylster). The approved method for euthanizing zebrafish is incubation in ice water.

Microinjections

Single cell zebrafish male and female embryos were injected with 200 pg of miR-153, 5 ng each of miR-153MO and miR-153loopMO and/or 100 pg of in vitro-transcribed, capped GFP reporter mRNA with or without the snap-25a or b 3’ UTR. Zebrafish snap-25a,b 3’ UTR sequences were amplified by PCR and subcloned downstream of the GFP ORF in pCS2+ [81].
Rescue experiments used injections of 3 ng of snap-25StartMO and snap-255’UTRMO, 150 pg of snap-25a,b mRNA, 250 pg of snap-25a mRNA, or 300 pg of snap-25b mRNA without 3’UTRs.

Two different morpholinos against miR-153 were utilized. One was perfectly complementary to the mature sequence; the second was complementary to a portion of the mature sequence and then extending into the precursor loop. Targeting of snap-25a,b mRNAs was performed using morpholinos against the region including the start codon.

Botulinum Toxin Analysis

Embryos injected at the 1-cell stage were treated with purified Botulinum neurotoxin A (Metabiologics, Inc., Madison, WI). Initial titration experiments were performed testing a range of BoNT A concentrations with final selection of 1 ng per 10 ml of water for 30 minutes at either 24-hpf or 48-hpf. Embryos were washed 10 times in fresh water and then allowed to recover for 1 hour prior to protein extraction or video capture to monitor movement.

qRT-PCR and Northern Blots

Total RNA extracted from both RFP+ and RFP- cells was reverse transcribed and qPCR reactions were carried out using Taqman miRNA assays (Life Technologies, NY) using the CFX96 Real-time PCR system (Bio-Rad), as previously described [32]. Northern blots were also performed as described [82,83].

Western Blots

Embryos were dechorionated, devolked, and sonicated in lysis buffer as described [83]. Approximately 100 embryos were pooled and one-tenth of the resulting samples were loaded into each lane. Membranes were probed with antibodies against α-tubulin (Abcam, ab15246), GFP (Torrey Pines, TP401) or SNAP-25 (Alomone Labs). For detection, anti-rabbit or anti-mouse HRP-conjugated secondary antibodies were used, followed by visualization with ECL.

GFP Reporter Analyses

Reporter analyses and western blots were as described [83]. To generate the snap-25a,b GFP reporters, the GFP ORF was fused to the 3’ UTR sequence of zebrafish snap-25a or b. snap-25a,b UTRs were cloned from zebrafish whole embryo RNA preparations using oligo d(T) primed reverse transcription followed by PCR amplification with gene specific primers. Images were acquired with a Leica MZFLII dissecting scope equipped with a fluorescent laser using a Qimaging camera with Qimaging software and imported into Adobe Photoshop for orientation and cropping.

Immunofluorescence

Embryos were fixed in 4% PFA overnight at 4°C and then permeabilized in 0.5% TritonX-100 for 60 minutes followed by treatment with protease K (20 μg/ul) for 10 minutes at room temperature. Samples were washed in PBT-DMSO before blocking overnight at 4°C (PBT-DMSO, 2% BSA, 5% goat serum). Primary antibodies (SNAP-25, 1:1000; SV-2, 1:300; ZNP-
1 mg/ml leupeptin (Worthington, NJ) and 100 U/ml DNaseI for 10 mins at room temperature and then kept on ice for RFP visualization, embryos were washed with PBT-DMSO, and then embryos were incubated with Cy3 or Cy5-conjugated donkey anti-mouse or rabbit antibodies (Jackson Immuno) for 4 hrs at room temperature. Before mounting and visualization, embryos were washed with PBT-DMSO. PC12 cells were fixed in 4% PFA for 15 mins, washed in PBS before incubating with primary antibodies for 1 hr, washed, incubated with secondary antibodies for 1 hr, Hoechst dye for 5 mins, washed, and visualized.

Tissue Dissociation and Motor Neuron Isolation

Tg(munc11-TagRFP-T) zebrafish embryos of 52 hpf were dechorionated, deyolked, and then dissected just posterior to the otic vesicle to collect trunks (excluding the hearts). Tissues were kept in buffer (1xPBS, pH 6.4, 1%BSA) and then dissociated using 16 U/ml papain and 0.2 U/ml Dispase (Worthington, NJ) for 30 mins at 28°C on a rotator. After complete dissociation of the tissue by careful pipetting up and down, cells were pelleted at 8000×g for 2 mins. Resuspended cells were then treated with 1 mg/ml leupeptin (Worthington, NJ) and 100 U/ml DNaseI (Sigma-Aldrich) in PBS at pH 7.4 containing 2 mg/ml MgCl2 for 10 mins at room temperature and then kept on ice for RFP+ and RFP− cell isolation. Gating was based on cell size and fluorescence intensity, determined by the control sample of dissociated cells from WT fish at the same developmental stage.

FM1-43 Dye Labeling

Embryos at 53 dpf were incubated in HBSS (137 mM NaCl, 5.4 mM KCl, 1 mM MgSO4, 0.44 mM KH2PO4, 0.25 mM Na2HPO4, 4.2 mM NaHCO3, 1.3 mM CaCl2, 5 mM Na-Ionated, deyolked, and then dissected just posterior to the otic vesicle to collect trunks (excluding the hearts). Tissues were kept in buffer (1xPBS, pH 6.4, 1%BSA) and then dissociated using 16 U/ml papain and 0.2 U/ml Dispase (Worthington, NJ) for 30 mins at 28°C on a rotator. After complete dissociation of the tissue by careful pipetting up and down, cells were pelleted at 8000×g for 2 mins. Resuspended cells were then treated with 1 mg/ml leupeptin (Worthington, NJ) and 100 U/ml DNaseI (Sigma-Aldrich) in PBS at pH 7.4 containing 2 mg/ml MgCl2 for 10 mins at room temperature and then kept on ice for RFP+ and RFP− cell isolation. Gating was based on cell size and fluorescence intensity, determined by the control sample of dissociated cells from WT fish at the same developmental stage.

Cell Culture and ELISA

PC12 cells (ATCC: CRL-1721) were maintained using Ham’s F12K media with 15% horse serum and 5% FBS, and transfected individually or in combination with miRNAs, mRNAs, and morpholinos. Transfections were performed with 300 nM miR-153, biotinylated snap-25 MOs and miR-153 MOs and 1.5 μg of snap-25a,b using Lipofectamine 2000 [84]. Co-transfection of a GFP plasmid was used to determine transfection efficiencies. Efficiencies less than 50% were discarded. One day after transfection, 50 ng/ml nerve growth factor was added to media to induce differentiation. Neurite outgrowth was assayed at day 5 by immunostaining with antibodies against acetylated α-tubulin. Stably transfected GH4C1 cells were a gift from Dr. K. Kannenberg [51]. ELISAs were performed after 5 days of transfection and human growth hormone was assayed following the Diagnostic Systems ELISA kit.

Supporting Information

Figure S1 Northern blot of miR-153 overexpression and knockdown. Perturbation of miR-153 expression levels by injection of miR-153 or MOs against different regions of pre-miR-153 was verified by northern blot. U6 served as a loading control.

Figure S2 Conservation of snap-25 3’ UTR sequences. The 3’ UTRs from mouse, human and zebrafish snap-25a (A) and snap-25b (B) are shown with the MREs that pair with miR-153 boxed in green. Conserved nucleotides are marked by an asterisk. The exact pairings between the MREs and miR-153 are shown in Figure 2 and Figure S3. Despite different levels of conservation, both MREs in snap-25a pair extensively with miR-153 in the seed region.

Figure S3 miR-153 targets snap-25b. (A) GFP reporter constructs were created by fusing the reading frame of GFP to the snap-25a 3’ UTR. Three predicted miRNA recognition elements (MREs) were identified in the snap-25b 3’ UTR. The miR-153 sequence is indicated in red and the corresponding snap-25a UTR sequence is shown in green. (B) Single cell zebrafish embryos were injected with mRNAs derived from GFP reporters lacking a UTR (GFP), fused to the full length snap-25b UTR (GFP+snap-25b), or mutant version of the snap-25a UTR lacking all MREs (GFP+snap-25b). Embryos were injected in the presence or absence of exogenous miR-153 or morpholinos against miR-153 (miR-153MO). Fluorescence levels were examined at 1 dpf. Clusters of embryos (~30) are shown. (C) Lysates from ~100 embryos were prepared from embryos treated as in B and GFP protein levels were determined by western blotting using antibodies against GFP or control antibodies against α-tubulin.

Figure S4 Dose-dependent rescue of miR-153 knockdown. (A) Single cell embryos were injected with a constant level of miR-153MO and increasing amounts (increments of 2 ng) of snap-25MO. Embryo lysates from ~60 embryos in each group were prepared and SNAP-25 protein levels determined by western blotting. (B) Quantitation of westerns (n = 3) from A. The grey circle represents the amount of snap-25MO (10 ng) used in co-injection rescue experiments.

Figure S5 Dose-dependent rescue of miR-153 overexpression. (A) Single cell embryos were injected with a constant level of miR-153 and increasing amounts (increments of 50 pg) of snap-25a, snap-25b, or snap-25a&b mRNA. Embryo lysates from ~60 embryos were prepared from embryos in each treatment group and SNAP-25 protein levels were determined by western blotting. (B) Quantitation of westerns (n = 3) from A. The grey circles represent the amounts used in co-injection rescue experiments (75 pg each of snap-25a and b, 250 pg of snap-25a, and 300 pg of snap-25b).

Figure S6 miR-153 regulates secondary motor neuron development. (A) Immunofluorescence was performed on whole mount zebrafish embryos at 53 hpf using Zn-α antibodies to label secondary motor neurons. Confocal images were acquired from the same somites for all embryos, as indicated. (B) miR-153 knockdown (miR-153MO) and snap-25a,b overexpression significantly increased the growth of secondary motor neuron axons (arrows). Overexpression of miR-153 or knockdown of snap-25a,b (snap-25a,bMO) caused severe defects in axon development and architecture (asterisks). Scale bar: 40 μm.

Movie S1 Embryo Movements in different conditions. 0:00–0:11. NIC Embryo Movements at 24 hpf. Noninjected control (NIC) zebrafish embryos at 24 hpf were filmed for one minute. Twitching was counted from individual embryos over multiple movies, as quantitated in Figure 1. 0:11–0:21. Effects of miR-153 knockdown.
of miR-153 Overexpression on Movement at 24 hpf Single cell zebrafish embryos were injected with miR-153 and filmed for one minute at 24 hpf. Twitching was counted from individual embryos over multiple movies, as quantitated in Figure 1. 0:22–0:32. Effects of Knockdown of miR-153 on Movement at 24 hpf Single cell zebrafish embryos were injected with miR-153(MO) and filmed for one minute at 24 hpf. Twitching was counted from individual embryos over multiple movies, as quantitated in Figure 1. 0:33–0:42. Effects of Decreased SNAP-25 Expression on Movement at 24 hpf Single cell zebrafish embryos were injected with snap-25a,b mRNA and filmed for one minute at 24 hpf. Twitching was counted from individual embryos over multiple movies, as quantitated in Figure 1. 0:42–0:52. Effects of Increased SNAP-25 Expression on Movement at 24 hpf Single cell zebrafish embryos were injected with snap-25a,b mRNA and filmed for one minute at 24 hpf. Twitching was counted from individual embryos over multiple movies, as quantitated in Figure 1. 1:02–1:12. Effects of co-Injection of miR-153 and snap-25a,b on Movement at 24 hpf Single cell zebrafish embryos were co-injected with miR-153 and snap-25a,b mRNA and filmed for one minute at 24 hpf. Twitching was counted from individual embryos over multiple movies, as quantitated in Figure 1. 1:12–1:22. NIC Embryo Movements at 28 hpf Noninjected control (NIC) zebrafish embryos at 28 hpf were filmed for one minute at 24 hpf. Twitching was counted from individual embryos, as quantitated in Figure 4C. 1:22–1:32. Effects of Botulinum Toxin Treatment on Movement at 28 hpf Single cell zebrafish embryos were injected with injection dye and treated with Botulinum toxin A at 27 hpf. After a 30 min treatment, embryos were washed and allowed to recuperate for 1 hour before being filmed. Twitching was counted from individual embryos, as quantitated in Figure 4C. 1:33–1:42. Effects of Botulinum Exposure and co-Injection of miR-153(MO) on Movement at 28 hpf Single cell zebrafish embryos were injected with miR-153(MO) and treated with Botulinum toxin A at 27 hpf. After a 30 min treatment, embryos were washed and allowed to recuperate for 1 hour before being filmed. Twitching was counted from individual embryos, as quantitated in Figure 4C. 1:42–1:52. Effects of Botulinum Exposure and co-Injection of snap-25a,b mRNA on Movement at 28 hpf Single cell zebrafish embryos were injected with snap-25a,b mRNA and treated with Botulinum toxin A at 27 hpf. After a 30 min treatment, embryos were washed and allowed to recuperate for 1 hour before being filmed. Twitching was counted from individual embryos, as quantitated in Figure 4C.

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Author Contributions

Conceived and designed the experiments: CW EJT AFO DJC ALP AFM. Performed the experiments: CW EJT JGP. Analyzed the data: CW EJT AFO BDC KB JGP. Contributed reagents/materials/analysis tools: CW EJT ALP. Wrote the paper: CW KB JGP.

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