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miR126-5p Downregulation Facilitates Axon Degeneration and NMJ Disruption via a Non-Cell-Autonomous Mechanism in ALS.

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Axon degeneration and disruption of neuromuscular junctions (NMJs) are key events in amyotrophic lateral sclerosis (ALS) pathology. Although the disease’s etiology is not fully understood, it is thought to involve a non–cell-autonomous mechanism and alterations in RNA metabolism. Here, we identified reduced levels of miR126-5p in presymptomatic ALS male mice models, and an increase in its targets: axon destabilizing Type 3 Semaphorins and their coreceptor Neuropilins. Using compartmentalized in vitro cocultures, we demonstrated that myocytes expressing diverse ALS-causing mutations promote axon degeneration and NMJ dysfunction, which were inhibited by applying Neuropilin1 blocking antibody. Finally, overexpressing miR126-5p is sufficient to transiently rescue axon degeneration and NMJ disruption both in vitro and in vivo. Thus, we demonstrate a novel mechanism underlying ALS pathology, in which alterations in miR126-5p facilitate a non–cell-autonomous mechanism of motor neuron degeneration in ALS.

**Key words:** ALS; axon degeneration; microfluidic chambers; miRNA; NMJ; Sema3A

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**Significance Statement**

Despite some progress, currently no effective treatment is available for amyotrophic lateral sclerosis (ALS). We suggest a novel regulatory role for miR126-5p in ALS and demonstrate, for the first time, a mechanism by which alterations in miR126-5p contribute to axon degeneration and NMJ disruption observed in ALS. We show that miR126-5p is altered in ALS models and that it can modulate Sema3 and NRP protein expression. Furthermore, NRP1 elevations in motor neurons and muscle secretion of Sema3A contribute to axon degeneration and NMJ disruption in ALS. Finally, overexpressing miR126-5p is sufficient to transiently rescue NMJ disruption and axon degeneration both in vitro and in vivo.
The degeneration that occurs in ALS is considered to be a non–cell-autonomous process involving interactions between the neuron and its diverse extracellular microenvironments via an unknown mechanism (Ilieva et al., 2009; Tsitkanou et al., 2016). Although the molecular basis for neuronal dysfunction and death in ALS is still poorly understood, it may be due to alterations in the nature of the extracellular signaling pathways that switch from prosurvival to toxic (Ilieva et al., 2009; Persson et al., 2009). Numerous studies support the notion that multiple tissues outside the CNS, including skeletal muscle (Dupuis et al., 2006; Tsitkanou et al., 2016), astrocytes (Nagai et al., 2007), and microglia (Lee et al., 2016), contribute to ALS pathologies. Alterations in RNA metabolism and microRNAs (miRs) can contribute to, and also be part of, mechanisms that initiate the disease (Lemmens et al., 2010; Emde and Hornstein, 2014). miRs are post-transcriptional regulators that play an important role in many cellular processes, such as axon growth and retraction, and were demonstrated to be involved in many diseases, including neurodegenerative diseases, such as ALS (Hawley et al., 2017; Molasy et al., 2017). Alterations in miR expression profile were identified specifically in axons of ALS models (Rotem et al., 2017), as well as in muscles leading to increasing attempts to either use or target miRs as therapeutic strategies (Di Pietro et al., 2017). Therefore, it is reasonable to assume that alterations in RNA and miRNA metabolism, of both MNs and neighboring cells, can regulate a secreted destabilizing signal, which in turn, facilitates axon degeneration and NMJ disruption.

Semaphorin3A (Sema3A) was initially identified as a repellent guidance molecule (Luo et al., 1993; Worzfeld and Offermanns, 2014). However, later works showed that it can also induce neuronal cell death of sympathetic, sensory, retinal, and cortical neurons (Nakamura et al., 2000; Shirvan et al., 2002; Ben-Zvi et al., 2008; Jiang et al., 2010). Neuruplin1 (NR1P1) has been shown to be the receptor binding component for Sema3A as well as some other Type 3 Semaphorins (Kolodkin et al., 1997). Sema3A was found to be upregulated following CNS injury as well as in several neurodegenerative diseases (Kaneko et al., 2006; Van Battum et al., 2015). Importantly, Sema3A was found to be upregulated in terminal Schwann cells of the SOD1G93A transgenic mouse model for ALS and in the motor cortex of ALS patients (De Winter et al., 2006; Körner et al., 2016), suggesting that it plays a toxic role in disease pathology and progression.

Here we demonstrated that alterations in miR126-5p result in upregulation of Type 3 Semaphorins and its cobinding receptor NR1P1 in muscles and MN axons of ALS models, respectively. We further demonstrate in vitro and in vivo the contribution of this pathway to axon degeneration and NMJ disruption in ALS models.
before sample donation, according to the guidelines of the Hospital’s Ethics Committee supervised by the Israeli Health Ministry Ethics Committee conforming with the Code of Ethics of the World Medical Association (Declaration of Helsinki), printed in the British Medical Journal (Rickham, 1964).

MN cell culture. Primary SC neurons were cultured using E12.5 mouse embryos of either sex as previously described (Zahavi et al., 2015). Briefly, SCs were excised, trypsinized, and triturated. Supernatant was collected and centrifuged through a 4% BSA cushion. The pellet was resuspended and centrifuged through an OptiPrep gradient (10.4% OptiPrep, Sigma-Aldrich, 10% v/v Tricine, 4% glucose) for 20 min at 760 g with the brake turned off. Cells were collected from the interface, washed once in complete medium, and then plated in coated growth chambers. Cells were maintained in Complete Neurobasal Medium (Invitrogen) containing B27 (Invitrogen), 10% (v/v) horse serum (Biological Industries), and 1% Glutamax (Invitrogen) supplemented with 1 ng/ml GDNF, 0.5 ng/mlCNTF, and 1 ng/ml BDNF (Alomone Labs). Before plating, the growth plates were coated with 1.5 mg/ml poly-γ-ornithine (Sigma-Aldrich) overnight at 37°C and 3 g/ml laminin (Sigma-Aldrich) for 2 h at 37°C. For immunofluorescence staining, 30,000 cells were plated on cover slides in 24-well plates. Cells were grown at 37°C in 5% CO₂.

SC explants. SCs were dissected from E11.5 mouse embryos of both sexes, either using HB9:GFP or SOD1G93A stripped of meninges and DRGs. The ventral horn was separated from the dorsal horn by longitudinal cuts along the SC, and transverse sections up to 1 mm were placed in the explant well. Before plating, the growth chambers were coated with 1.5 mg/ml poly-γ-ornithine overnight at 37°C and 3 g/ml laminin overnight at 37°C. Explants were maintained in Spinal Cord Explant Medium containing Neurobasal, 2% B27, 1% PS, and 1% Glutamax, supplemented with 25 ng/ml BDNF. Explants were grown at 37°C in 5% CO₂.

Primary myocyte culture. Skeletal muscle cultures were derived from the GC muscle of adult P60 female mice of either SOD1G93A background or their littermates (LMs) using techniques previously described (Ionescu et al., 2016). Briefly, GC muscles were excised and incubated in 2 mg/ml collagenase 1 (Sigma-Aldrich) in DMEM containing 2.5% PSN (Biological Industries) for 3 h. Muscles were then dissociated and incubated for 3 d in Matrigel-coated (BD Biosciences) 6-well plates with Bioamf-2 medium (Biological Industries) with 1% PSN at a density of ~120 myofibers per well. For purification of the myoblasts, adherent cells were trypsinized and preplated in an uncoated dish for 1 h at 37°C. Nonadherent cells were then transferred into a Matrigel-coated dish with Bioamf-2 medium. Preplating was repeated for 2 d, keeping the culture at ~<50% confluence, before plating cells in MFC. Cultures were maintained at 37°C and in 5% CO₂. After the final preplating, 100,000 myocytes were post-plated method and a mixed consisting of the ventral of Myocyte conditioned media (CM) were produced as follows: At the final preplating stage, myoblasts were cultured in a Matrigel-coated 100 mm dish at 80% confluence and were incubated for 2 d with Bioamf-2 medium, followed by 2 d with rich DMEM (Biological Industries) medium containing 10% FCS (Biological Industries), 10% horse serum (Biological Industries), 1% Glutamax, and 1% PSN. Then, once muscles reached a fully differentiated state, the culture dish was rinsed 3 times with preheated PBS and poor DMEM containing 1% Glutamax, and 1% PSN was applied on the cultures. CM was collected after 2 d, centrifuged for 5 min at 400 × g at 25°C, and streamed through a 0.22 μm PES filter.

CM preparation and application. Muscle myocytes of WT or SOD1G93A mice were cultured as described previously (Ionescu et al., 2016). Seven days after myocytes were fully differentiated, the muscles kept growing for 3 d in complete Neurobasal containing BDNF and GDNF. The CM was refreshed with BDNF, GDNF, and glucose after its collection, as described previously (Nagai et al., 2007). CM under both conditions was applied on the axon compartment of the MFC for 48 h.

Lentiviral vectors. Genes of interest were cloned into a third-generation lentiviral pLLE3.7 backbone. HEK293T cells were transfected by using calcium phosphate precipitation. Lentiviral vectors were produced by NanoString Technologies’ facilities for an miR-Chip array assay of ~800 known miRs (and incubated chromatography at 37°C). Samples were quantified automatically by NanoString Technologies’ instrumentation for miRs, which was hybridized with the template. Output data were analyzed by the
nCounter analysis system. All miRs were normalized to the 100 most abundant miRs in the samples.

**Primer design.** Based on the consensus sequences of the desired transcripts, 2 sets of primers were designed for each gene (Table 1). qPCR for miRNA detection. qPCR was performed on the StepOne system (Invitrogen) in a 10 µl reaction containing 4 µl of RNA (20 ng), 5 µl Syber green master mix (Thermo Fisher Scientific), and 1 µl of reverse and forward primers.

**miR vectors and transfection.** Mammalian expression vector pMSCV-Blast-miR constructs were generously provided by Erin Hornstein (Weizmann Institute of Science). Mammalian expression vector of C9orf72 Di-peptide PK30 and GK30 constructs was generously provided by David Trots (Jefferson University) (Wen et al., 2014). Next, 50,000 HeLa/U87 human glioblastoma/muscles cells were plated in rich DMEM (1% PS, 1% GlutaMAX). After 24 h, the culture medium was replaced with serum-free medium (Opti-MEM), and cells were transfected using FuGene NE 6 (Promega) protocol. Cells were collected after 48 h and used either for a functional assay or for RNA/protein extracts.

**Semaphorin preparation.** HEK293T cells were stably transfected to overexpress either Sema3A or an empty control. CM from 80% confluent cultures were collected after 3 d. We validated the purity level of the collected media using Coomassie staining and identified the stained band with a specific antibody against the desired protein using Western blot analysis.

**NRP1 antibody application.** A total of 5 µg/ml NRP1 antibody (R&D System, AF566 dot ETH0915031) for the extracellular domain was added to the distal compartment of the MFC while maintaining a proximal-to-distal volume gradient.

**Histology tissue collection and fixation.** GC muscles of 20 samples were harvested and fixed in 4% PFA. The samples were then outsourced for a histological assessment at Patho-Logica. All tissues were trimmed into block cassettes and sent to CDX Diagnostics for slide preparation. Slide preparation and historical evaluation. Tissues were trimmed, embedded in paraffin sections at no more than 5 µm thickness, and stained with H&E. The mean minimal muscle fiber diameter thickness was measured in microns by performing a manual count using a 10x lens and analyzed by expert pathologist.

**xCELLigence impedance measurement.** For each experiment, 30,000 U87 cells were plated with rich DMEM in E-Plate L8 wells and incubated together with the xCELLigence system (ACEA Biosciences) at 37°C, 5% CO2 overnight. Impedance data were collected at 5 min intervals. After 24 h, poor DMEM (1% PS, 1% GlutaMAX) with Sema3A or its control medium was replaced and recording proceeded. The data were analyzed using RTCA data analysis software 1.0 and normalized to the control sample.

**CatWalk XT gait analysis.** The CatWalk is a video-based analysis system used to assess gait in voluntarily walking mice (Noldus Information Technology). The principle of this method is based on an optical technique. The light of a fluorescence tube is completely internally reflected on a glass walkway floor. When the animal crosses the walkway, the light leaves the glass and illuminates only the area of contact. In this way, the different paw contacts are visualized. Based on position, pressure, and the surface area of each foot paw, multiple parameters are calculated.

Only compliant and continuing trials for each animal were analyzed, averaged, and the mean was calculated.

**Results**

**Sema3A and NRP1 levels are elevated in muscles and the MNs of ALS models**

ALS disease is considered to be a distal axonopathy involving axon degeneration and NMJ disruption as key processes in its pathology (Fischer et al., 2004). We therefore hypothesized that destabilizing factors secreted from adult presymptomatic ALS mutant muscles might be involved in triggering axon degeneration of MNs. Because Sema3A is known to act in such a manner, at least in development, and it was already reported to be elevated in ALS, we decided to focus on this factor (De Winter et al., 2006; Körner et al., 2016). Following this hypothesis, we first examined the expression of Sema3A in SOD1G93A GC muscles in comparison with that of their LMs (Fig. 1A, B, Fig. 1-1, available at https://doi.org/10.1523/JNEUROSCI.3037-17.2018.61-1). Western blot analysis of muscle protein extracts revealed significant elevations in Sema3A protein levels in muscles of presymptomatic SOD1G93A mice as early as P30 and P60, whereas testing Sema3A levels in younger animals (P7) showed no apparent differences compared with their LM controls (mean fold change over LM: P30 SOD1G93A, 3.08 ± 0.86; P30 LM, 1 ± 0.36; P60 SOD1G93A, 2.2 ± 0.45; P60 LM, 1 ± 0.32; P7 SOD1G93A, 1.2 ± 0.7; P7 LM, 1 ± 0.42). To validate this difference, we also tested the transcription levels of Sema3A (Fig. 1C). qPCR analysis of total RNA extracts from muscles at presymptomatic SOD1G93A stage and LM mice identified an ~1.7-fold increase in Sema3A mRNA of SOD1G93A muscles (mean fold change over LM: SOD1G93A, 1.72 ± 0.32; LM, 1 ± 0.06). Because GC muscle tissues contain heterogeneous cell types and to verify that the levels of Sema3A are indeed higher specifically in SOD1G93A muscles fibers, we immunostained primary myocyte cultures from P60 SOD1G93A and LM mice for Sema3A (Fig. 1D, E). Quantifying the mean intensity values showed a significant 50% increase in the SOD1G93A myocytes (a mean fold change in intensity over LM: SOD1G93A, 1.5 ± 0.06; LM, 1 ± 0.04). We also collected CM from myocyte cultures to determine whether the increase in Sema3A protein also results in an increase in its secretion (Fig. 1F). Western blot analysis indicated that Sema3A levels were also elevated in P60 SOD1G93A myocyte-CM (a mean fold change over LM: SOD1G93A, 2.3 ± 0.55; LM, 1 ± 0.08). Because NMJ disruption is a primary event in ALS, we sought to examine the levels of Sema3A in NMJ in vivo (Fig. 1G, H). Immunostaining for Sema3A in GC muscles showed a sixfold increase in the number of muscle fibers expressing Sema3A in their NMJs. Whereas only ~5% of NMJs stained positively for Sema3A in LM muscles, we identified its expression in ~30% of NMJs in P60 SOD1G93A mice (the mean percentage of NMJs expressing Sema3A: SOD1G93A, 30.83 ± 4.73%; LM, 4.56 ± 2.4%). Interestingly, a previous study described Sema3A elevation in SOD1G93A mice specifically in fast-fatigue NMJs expressing myosin-Iib marker (De Winter et al., 2006). Because fast fatigue NMJs are the first to become disrupted and be eliminated in ALS pathology, we examined Sema3A levels both at P90 and P120 and hypothesized that Sema3A levels will eventually drop in later stages of the disease. We found that whereas the percentage of NMJs expressing Sema3A in SOD1G93A in P90 animals is similar to P60, the end-stage animals (P120) were shown to display a reduction in Sema3A-positive NMJs, and
Figure 1. Presymptomatic elevation in the levels of Sema3A and NRP1 in ALS models. A, B, Western blot analysis of P30 and P60 GC muscle extracts revealed that the levels of Sema3A are elevated in presymptomatic SOD1<sup>G93A</sup> muscles compared with their corresponding LM control wherein at earlier stages we found no significant difference (Figure 1-1, available at https://doi.org/10.1523/JNEUROSCI.3037-17.2018.f1-1). Tubulin was used as a loading control. P30 (Student’s t test, n = 3, *p = 0.042). P60 (Student’s t test, n = 4, *p = 0.038). C, qPCR analysis of presymptomatic P60 and P30 GC muscle extracts also shows an elevation in the mRNA levels of Sema3A in SOD1<sup>G93A</sup> (Student’s t test, SOD1<sup>G93A</sup>, n = 5, LM, n = 4, *p = 0.049). D, Immunostaining of primary myocytes after 7 d in culture shows elevated levels of Sema3A in primary myocytes of SOD1<sup>G93A</sup>. White represents Sema3A. Blue represents nuclear DAPI staining. Scale bars, (Figure legend continues.)
no apparent difference existed between WT and SOD1<sup>G93A</sup> mice (Fig. 1-2A, B, available at https://doi.org/10.1523/JNEUROSCI.3037-17.2018.f1-2). Together, these results indicate that a significant part of the MN population is exposed to high levels of Sema3A in presymptomatic stages and that this specific population is disrupted and eliminated during disease progression. We then proceeded to investigate the expression of the Sema3A-receptor binding unit, NRPI, in ALS (Fig. 1f). Western blot analysis of GC muscle extracts revealed a significant ~8-fold increase in NRPI (the mean fold change over LM: SOD1<sup>G93A</sup>, 8.6 ± 2.2; LM, 1 ± 0.3). Because MNs are a primary target in ALS, we wanted to determine whether NRPI is also overexpressed in the MNs of SOD1<sup>G93A</sup> mice. First, we performed Western blot analysis of SNs and observed an ~2-fold elevation in NRPI levels of P60 SOD1<sup>G93A</sup> mice (Fig. 1f; the mean fold change over LM: SOD1<sup>G93A</sup>, 1.96 ± 1.22; LM, 1 ± 0.21). Next, we obtained protein extracts of primary MN cultures for Western blot analysis and confirmed an ~2.5-fold elevation in NRPI levels in the MNs of SOD1<sup>G93A</sup> culture (Fig. 1k; the mean fold change over LM: SOD1<sup>G93A</sup>, 2.3 ± 0.16; LM, 1 ± 0.06). Immunostaining of primary MN cultures for NRPI resulted in analogous findings (Fig. 1L–N). Intriguingly, the NRPI signal in SOD1<sup>G93A</sup> is generally higher than in LM and is increased even more in axons compared with cell bodies (the mean fold change over LM: SOD1<sup>G93A</sup> soma, 1.86 ± 0.13; LM soma, 1 ± 0.5; SOD1<sup>G93A</sup> axon, 3.83 ± 0.95; LM axon, 1 ± 0.11). Finally, immunostaining for NRPI in GC muscles confirmed a similar shift of ~30% in the number of NMJs expressing NRPI, as we had observed for Sema3A in SOD1<sup>G93A</sup> mice, both at P60 and P90. However, also this time, the differences were abolished in the end stages of the disease (P120) (Fig. 1O,P; Fig. 1-2C,D, available at https://doi.org/10.1523/JNEUROSCI.3037-17.2018.f1-2; the mean percentage of NMJs expressing NRPI: P60: SOD1<sup>G93A</sup>, 27.5 ± 2.04%; LM, 21.27 ± 1.22%). To determine whether the elevated NRPI levels result from feedback due to an increase in its ligand, we treated primary MN cultures from LM embryos with soluble Sema3A for 3 d and performed Western blot analysis on cell culture lysates. Importantly, we did not observe any difference in NRPI expression after applying Sema3A, suggesting that NRPI levels are regulated by an intrinsic mechanism in MNs (Fig. 1-3, available at https://doi.org/10.1523/JNEUROSCI.3037-17.2018.f1-3). Finally, to validate our finding with other ALS models and to emphasize the impact of Sema3A in ALS, we performed Western blot analysis for Sema3A and NRPI expression in human mesenchymal stem cells from sporadic ALS patients and healthy controls, as well as in myocyte-expressing C9orf72-PR50 and their CM for Sema3A. In addition, we compared the results with those of a mock control. Remarkably, in all of these ALS models, we identified high expression of Sema3A and NRPI (Fig. 1-4, available at https://doi.org/10.1523/JNEUROSCI.3037-17.2018.f1-4).

Together, our combined in vivo and in vitro data suggest that the levels of both Sema3A and its co-receptor NRPI, are presymptomatically increased in several ALS models as well as in sALS patients. These findings suggest that the Sema3A pathway is a common denominator in various ALS mutations; thus, it may contribute to MN degeneration in ALS.

**Application of Sema3A on wild-type MN axons results in axon degeneration**

Because our findings suggest that Sema3A is produced and secreted in excess from muscles of ALS models, and because muscles interact specifically with MN axons, we sought to test the activity of Sema3A exclusively in this distal subcellular compartment. To this end, we used an MFC that allows precise control, monitoring, and manipulation of subcellular microenvironments (Fig. 2-1, available at https://doi.org/10.1523/JNEUROSCI.3037-17.2018.f2-1) (Zahavi et al., 2015). We cultured healthy ventral SC explants from transgenic mouse embryos expressing GFP under the MN-specific promoter HB9 (HB9:GFP) in one compartment of the MFC and enabled axons to extend into the opposing compartment, thus segregating axons and cell bodies into two isolated compartments. To verify that our MFCs can efficiently segregate MN axons from their somata, we stained the neuronal culture in the MFC system for the dendritic and axonal markers MAP2 and Tau, respectively (Fig. 2-1, available at https://doi.org/10.1523/JNEUROSCI.3037-17.2018.f2-1). We confirmed that all neurites that traversed the distal compartment are positive for Tau staining and negative for MAP2. Next, we purified Sema3A or control media as described previously (Ben-Zvi et al., 2006), and applied them to the distal compartment, while imaging the axons for 16 h (Fig. 2A). Our recordings reveal extensive axon degeneration in the Sema3A-treated MFCs 6–8 h after its application (Fig. 2B; Movies 1, 2; the mean percentage of degenerated axons: Sema3A, 83.01 ± 3.54%; control, 23.94 ± 7.6%). Coapplication of NRPI-blocking antibody and Sema3A on MN axons inhibits the Sema3A-dependent axon degeneration (Fig. 2B; the mean percentage of degenerated axons: Sema3A and NRPI antibody, 25.00 ± 12%). These data indicate that Sema3A can trigger axon degeneration in MNs when applied exclusively on distal axons, and further support our hypothesis that an increase in muscle-derived Sema3A might contribute to axon degeneration in ALS.
Figure 2. Sema3A as well as primary myocytes expressing diverse ALS-causing mutations impair the growth of wild-type HB9::GFP motor axons and enhance their retraction and degeneration. A, Experimental procedure illustration and representative time-lapse images of HB9::GFP motor axons (Figure 2-1, available at https://doi.org/10.1523/JNEUROSCI.3037-17.2018.f2-1) in the distal compartment of an MFC with no muscles after applying Sema3A to the distal compartment. After 6 h, axons in the distal compartment of chambers that were treated with Sema3A undergo degeneration, whereas axons in the control chamber or axons cotreated with NRP1 antibody and Sema3A continue growing. Scale bar, 20 μm. B, Quantification of the rate of degraded axons in the distal compartment revealed a higher percentage of degradation in chambers that were exposed to Sema3A compared with either control or coapplication of Sema3A and NRP1 antibody (60 axons for Sema3A treatment, 70 axons for Control; Student's t test; n = 4; mean ± SEM, ***p = 0.00022). C, Schematic view of the experimental procedure in D–F. HB9::GFP SC explants and primary myocytes of SOD1G93A, TDP43A315T, C9orf72-PR50, C9orf72-GR50, or LM, GFP, and SOD1wt as controls were cocultured in an MFC (Figure 2-2, available at https://doi.org/10.1523/JNEUROSCI.3037-17.2018.f2-2), and the growth of HB9::GFP axons was assessed by time-lapse imaging of the microgroove compartment. D, Representative time-lapse images of the HB9::GFP axon growth when cocultured with (left to right) LM, SOD1G93A, and SOD1G93A + NRP1 antibody. The presence of SOD1G93A myocytes in the distal compartment triggers the retraction and degeneration of HB9::GFP motor axons growing in the groove compartment and prevents their traversing. When NRP1 antibody is applied to the distal compartment, together with SOD1G93A-expressing myocytes, axons are less prone to degenerate. Scale bar, 5 μm. E, Quantification of the rate of axons traversing the distal compartment in B shows the mean percentage of axons that traversed the distal compartment out of the total axons in each field (n = 3; NRP1 antibody experiment, n = 4; Student’s t test, *p = 0.025, **p = 0.0433). F, Quantification of (Figure legend continues.)
Muscles expressing diverse ALS mutations initiate axon degeneration

To study the molecular mechanisms enabling the communication between MNs and their environment, which are essential for cell survival and synapse maintenance, we extended the use of the MFC system to coculture primary MNs and primary myocytes (Ionescu et al., 2016). Briefly, ventral SC explants from healthy 12-day-old (E12) HB9::GFP embryos were cultured in the proximal compartment, in the presence or absence of primary myocytes extracted from adult mice in the distal compartment (Fig. 2-1, available at https://doi.org/10.1523/JNEUROSCI.3037-17.2018.f2-1). As we showed previously (Zahavi et al., 2015), culturing HB9::GFP explants in the presence of wild-type muscles facilitates the rapid and directed growth of axons into the distal compartment (Fig. 2-1, available at https://doi.org/10.1523/JNEUROSCI.3037-17.2018.f2-1), suggesting that muscles secrete factors that support and promote the growth of motor axons. However, because ALS-mutated muscles were found to have intrinsic abnormalities throughout disease progression (Loeffler et al., 2016), we hypothesized that the nature of these factors will be altered. To study the effect of ALS muscles on MN axons in a simplified system, we plated primary myocytes from presymptomatic P60 SOD1G93A and LM mice as well as WT myocytes transfected to express SOD1wt in the distal compartment. Myocyte cultures were allowed to fuse and differentiate. Importantly, in all the described cases, myocyte morphology, fusion, and differentiation parameters were similar, and the culture showed no apparent difference (Fig. 2-2, available at https://doi.org/10.1523/JNEUROSCI.3037-17.2018.f2-2). After 7 d, HB9::GFP SC explants were cultured in the proximal compartment. Cocultures were incubated until the HB9::GFP axons began extending toward the microgroove compartment. Once the axons reached the microgroove compartment, the extension of HB9::GFP axons along the microgrooves was recorded for 16 h.
whether Type 3 Semaphorins, such as Sema3A, contribute to MN axon degeneration in this assay, we investigated whether NRP1-blocking antibody application can block this phenotype. Here again, we observed a rescue effect by this treatment (Fig. 2H; SOD1G93A + NRP1-antibody 18.6 ± 7%; although the protection was incomplete. These results reinforce our hypothesis, suggesting that ALS-mutated muscles secrete destabilizing factors, such as Sema3A. Importantly, these results emphasize that SOD1G93A MNs exhibit a higher sensitivity to degeneration, and support the MN unique vulnerability as well as the non–cell-autonomous mechanism of ALS. Interestingly, previous attempts to block Sema3A signaling in SOD1G93A mice using either a similar antibody approach or by crossing transgenic mice expressing a truncated form of Sema3A with SOD1G93A mice also resulted in only a mild improvement or none at all of motor functions (Venkova et al., 2014; Moloney et al., 2017). This suggests that Sema3A plays a complex role in MNs and that perhaps other related proteins are involved. This also led us to investigate whether a wider deregulation of secreted factors released by the diseased muscles exists. Indeed, examining other members of the Semaphorin family, we found that the percentage of NMJs expressing Sema3B, as well as NR2P2 is elevated in the SOD1G93A ALS model (Fig. 2-4, available at https://doi.org/10.1523/JNEUROSCI.3037-17.2018.f2-4). Therefore, we concluded that the destabilizing effect of ALS muscles over MN axons involves more than a single factor; thus, it cannot be blocked or rescued by targeting one factor at a time. Moreover, the multiplicity of effectors suggests that a higher-order regulator, such as miRNA, might be involved in this process.

miR126-5p is downregulated in ALS models and modulates Sema3A, Sema3B, NRP1, and NR2P2 protein expression levels

To identify the mechanism underlying the elevated levels of various secreted destabilizing factors in muscles of ALS models, we scanned for alterations in miRNAs (miRs) that can regulate the expression of multiple proteins. miRs have been previously linked to MN toxicity in ALS (Haramati et al., 2010). We used Nanostring miRNA-chip technology to screen for alterations in ~800 miRs of presymptomatic P60 SOD1G93A mice and their LM controls. The screen yielded 8 significantly altered miRs (Fig. 3A; Fig. 3-1, available at https://doi.org/10.1523/JNEUROSCI.3037-17.2018.f3-1). Because we found that Sema3A levels were elevated in muscles, we narrowed our focus to those miRs that were reduced and that could regulate its expression, specifically miR126-5p and miR133a (Fig. 3B; the mean fold change over LM: SOD1G93A, 0.74 ± 0.03; LM, 1 ± 0.03; Fig. 3-1, available at https://doi.org/10.1523/JNEUROSCI.3037-17.2018.f3-1). A targeted search for these miRs in databases (miRDB, Pictar, miRbase, and miRTarBase) revealed that both miRs are predicted to regulate Semaphorin signaling genes as well as other relevant transcripts of ALS-related genes. Interestingly, we previously described deep-sequencing analyses of primary MN cultures expressing SOD1G93A or TDP43AS1 and demonstrated that miR126-5p is also correspondingly decreased in diseased MN axons, but not in their soma (Rotem et al., 2017). This information led us to further focus our investigation on miR126-5p. We used qPCR to validate that miR-126–5p levels in SOD1G93A GC muscles point to a similar trend (Fig. 3C; the mean fold change over LM: SOD1G93A, 0.47 ± 0.2; LM, 1 ± 0.45). To verify that miR126-5p can regulate the expression of Semaphorin3 and Neuropilin signaling members, we transfected HeLa cells, which are known to endogenously express Sema3A, Sema3B, NRP1, and NRP2 (Fujita et al., 2001), with miR126-5p or with the irrelevant miR142, which is not predicted to target any of these genes,
as a negative control. To this end, we isolated RNA from these cultures and performed qPCR analysis to determine the mRNA levels of Sema3A, Sema3B, NRP1, and NRP2 (Fig. 3D–G). Our results indicate that miR126-5p specifically targets Sema3A, NRP1, Sema3B, and NRP2 (RT-PCR: mean Ct-NRP1: miR126, 3.79 \pm 0.71; miR142, 2.83 \pm 0.57; ΔCt-Sema3A: miR126, 4.84 \pm 0.22; miR142, 3.84 \pm 0.34; ΔCt-NRP2: miR126, 7.6 \pm 0.30; miR142, 6.2 \pm 0.37; ΔCt-Sema3B: miR126, 8.1 \pm 0.10; miR142, 7.05 \pm 0.14). To investigate whether miR126-5p overexpression can also inhibit Sema3A function, we used a recently described impedance-based approach. U87MG human glioblastoma cells, which express NRP1 (Fig. 3D–G, available at https://doi.org/10.1523/JNEUROSCI.3037-17.2018.f3-2), were transfected to overexpress miR126-5p or miR142 as a control. One day after transfection, cells were resuspended and plated in xCELLigence multwell electric plates. The next day, Sema3A was added to the cultures, and any morphological or adhesive changes were monitored by the impedance readout. As demonstrated by TIRF imaging (Fig. 3H), adding Sema3A to responsive cells, such as U87MG cells, induces their detachment from the culture dish. This detachment can be measured as a decrease in impedance (Fig. 3-2B, available at https://doi.org/10.1523/JNEUROSCI.3037-17.2018.f3-2). Shortly after Sema3A was added to the cultures, cells expressing miR142 exhibited decreased impedance, whereas cells expressing miR126-5p did not respond to Sema3A in the medium and kept growing with a corresponding increase in impedance (Fig. 3I). Hence, we showed that the excess production of destabilizing factors in ALS is likely to be mediated downstream of a deregulation in miR126-5p.
Overexpression of miR126-5p can block SOD1<sup>G93A</sup> muscle toxicity

We overexpressed miR126-5p in SOD1<sup>G93A</sup> myocyte cultures and quantified Sema3A levels in their culture extract as well as in their CM. Western blot analysis indicated that Sema3A levels in both the culture extract and CM are depleted, compared with miR142 (Fig. 4A, B; the mean fold change over SOD1<sup>miR142</sup>: Muscle extract-SOD1<sup>miR126</sup>, 0.24 ± 0.1; SOD1<sup>miR142</sup>, 1 ± 0.4 Muscle-CM-SOD1<sup>miR126</sup>, 0.63 ± 0.03; SOD1<sup>miR142</sup>, 1 ± 0.13). Next, we investigated whether overexpressing miR126-5p in both SOD1<sup>G93A</sup> and PR50 myocytes can rescue the negative effect on MN growth that we observed before. To this end, primary myo-
miR126-5p transiently rescues early motor phenotypes of 
\textit{SOD1}^{G93A} \textit{mice in vivo}

NMJ disruption, muscle morphology abnormalities, and hindlimb misprints are major phenotypes in \textit{SOD1}^{G93A} \textit{mice} (Gurney et al., 1994). To determine whether miR126-5p can moderate those phenotypes, we injected \textit{SOD1}^{G93A} \textit{mice} with either pLL-eGFP-miR126 (SOD1\textit{miR}^{126}) or pLL-eGFP-miR142 (SOD1\textit{miR}^{142}) into the right and left GC muscles of presymptomatic mice (P60), respectively (Fig. 5A). Virus expression was validated both in vitro on MNs and in muscle cultures (Fig. 5-1A, available at https://doi.org/10.1523/JNEUROSCI.3037-17.2018.f5-1) as well as in vivo at the transcript and protein levels (Fig. 5-1 B, C, available at https://doi.org/10.1523/JNEUROSCI.3037-17.2018.f5-1). Importantly, we observed a decrease in the number of NMJs expressing Sema3A in the pLL-eGFP-miR126-5p-injected GC muscles in comparison with the miR142 group, suggesting that miR-126-5p is active in the injected tissue (Fig. 5-1 D, available at https://doi.org/10.1523/JNEUROSCI.3037-17.2018.f5-1).

Next, we performed a series of histological analyses, followed by motor behavioral tests at two time points after injection: at the age at which mice typically begin exhibiting ALS phenotypes (P90) as well as in the disease end stage (P120) (Fischer et al., 2004). Because NMJ disruption is a key process in ALS, we sought to determine whether overexpression of miR126-5p results in reduced NMJ disruption. Briefly, both the left and right GC muscles were fixed and stained for synaptic markers of the NMJ (Fig. 5B). Quantifying the percentage of intact NMJs at P90 injected mice revealed a significantly higher innervation rate in miR126-5p-expressing muscles compared with both mock-treated and \textit{SOD1}^{G93A} \textit{mice} (Fig. 5C; P90: WT, 71.58 \pm 3.32%; \textit{SOD1}^{G93A}, 42.58 \pm 2.64%; SOD1\textit{miR}^{126}, 64.25 \pm 5.88%; SOD1\textit{miR}^{142}, 46.54 \pm 7.2%). Furthermore, careful analysis at P120 also identified a mild rescue by miR-126-5p overexpression. (Fig. 5C; P120: WT, 74.35 \pm 4.74%; \textit{SOD1}^{G93A}, 20.12 \pm 5.01%; SOD1\textit{miR}^{126}, 30.82 \pm 3.97%; SOD1\textit{miR}^{142}, 18.18 \pm 3.12%). Next, we performed histological analyses to determine muscle fiber wasting and tissue abnormalities (Fig. 5D, E). P120 GC muscles of \textit{WT}, \textit{SOD1}^{G93A}, and both SOD1\textit{miR}^{126} and SOD1\textit{miR}^{142} were stained with H&E for histological examination, and the minimal diameter of myofibers was analyzed as described in Materials and Methods. We observed a mild, but significant, increase in the minimal fiber size of the SOD1\textit{miR}^{126}-injected muscle compared with the SOD1\textit{miR}^{142} mock control (Fig. 5G; Fig. 5C; P120: WT, 40.25 \pm 2.28%; \textit{SOD1}^{G93A}, 18.5 \pm 0.64%; SOD1\textit{miR}^{126}, 23 \pm 1.87; SOD1\textit{miR}^{142}, 19 \pm 1.47). Last, we performed a behavioral test using the CatWalk gait analysis technique. This video-based method is a computerized version of the ink bath assay and provides an objective and dynamic wide range of gait analyses (Deumens et al., 2007). Moreover, it has been used before specifically with the \textit{SOD1}^{G93A} mouse model and displayed significant differences in several parameters (Mead et al., 2011; Gerber et al., 2012) (Fig. 5F). One output is the Mean Stand Index (MSI), which measures the speed at which the paws detach from the walking surface. Because aged \textit{SOD1}^{G93A} mice suffer motor defects, their MSI values for both hindlimbs are dramatically lower than their LM values. Remarkably, the MSI values of the SOD1\textit{miR}^{126}-injected limbs were significantly higher at P90 and similar to the LM control values, whereas the SOD1\textit{miR}^{142}-injected limb was reminiscent of typical \textit{SOD1}^{G93A} behavior (Fig. 5G; mean fold change over WT: \textit{SOD1}^{G93A}, 0.68 \pm 0.02; SOD1\textit{miR}^{126}, 0.74 \pm 0.06; SOD1\textit{miR}^{142}, 0.65 \pm 0.04; LM, 1 \pm 0.04). We also examined other established parameters that have been shown to be altered in the \textit{SOD1}^{G93A} model (Mead et al., 2011). We specifically focused on the percentage of single-support parameter, which indicates the relative duration of contact of all combined paws with the glass floor, and on the base of support parameter, which indicates the average width of limb spreading between both front, or both hindpaws. Remarkably, we observed a significant rescue phenotype for both parameters in the injected mice at age of P90.
Figure 5. pLL-eGFP-miR126-5p injected into GC muscles of presymptomatic SOD1G93A mice transiently rescues the early phenotype appearance in vivo. A, Schematic view of the in vivo experimental procedure. SOD1G93A mice were injected with either pLL-eGFP-miR126-5p or pLL-eGFP-miR142 in their right or left GC muscles, respectively. Viral infection was validated (Figure 5-1, available at https://doi.org/10.1523/JNEUROSCI.3037-17.2018.f5-1). B, Representative whole-mount NMJ immunostaining of P90 SOD1G93A GC muscles injected with either miR126-5p or miR142 lenti vectors. Red represents BTX. Green represents NFH/synaptophysin in presynaptic neurons. Scale bar, 20 μm. C, The percentage of innervated NMJs in miR126-5p-injected muscles is higher compared with its controls in both P90 and P120 (P90: Student’s t test, n = 6, *p = 0.0475, **p = 0.001245; P120: Student’s t test, n = 5, *p = 0.043, **p = 0.0096). D, Representative histological images of P120 WT, SOD1G93A, miR126-5p, and miR142 GC muscle cross sections after H&E staining. Scale bar, 100 μm. E, Semiquantification of a GC cross section from D shows a significant increase in the minimal muscle fiber diameter of muscles that were injected with miR126-5p (P120: Student’s t test, n = 4, *p = 0.031). F, Illustration of the CatWalk XT gait analysis system that monitors mouse footprints. G, Gait analysis MSI parameter indicates the speed at which the paw loses contact with the surface. The MSI for the P90 miR126-5p-injected limbs was significantly higher than for miR142-injected limbs (Student’s t test, *p = 0.0355). H, Gait analysis percentage single-support parameter indicates the relative duration of contact of a single paw on the glass floor. The percentage in which the injected animals were used along the run with a single paw was significantly higher compared with SOD1G93A mice and showed similarity to the WT control (Student’s t test, SOD1G93A-injected, ***p = 0.0004; WT-SOD1G93A, ***p = 0.000003). I, Gait analysis base of support parameter indicates the average width between the hindpaws. The base of support of both P90- and P120-injected mice was significantly higher compared with SOD1G93A (Student’s t test, P90SOD1G93A-injected, ***p = 0.000006; WT-SOD1G93A, ***p = 0.000007; P120 SOD1G93A-injected, ***p = 0.000003; WT-SOD1G93A, ***p = 0.00000009).
Furthermore, the improvement in base of support parameter persisted also in P120 (Fig. 5H, I; Percentage of Support Single: SOD1\textsuperscript{G93A}, 0.43 ± 0.05; injected, 1.06 ± 0.24; LM, 1 ± 0.1; Base of Support, P90: SOD1\textsuperscript{G93A}, 0.87 ± 0.01; injected, 1.02 ± 0.02; LM, 1 ± 0.02; P120: SOD1\textsuperscript{G93A}, 0.858 ± 0.02; injected, 0.95 ± 0.02; LM, 1 ± 0.02).

Together, we demonstrated in vivo that miR126-5p reduces the detrimental effects of muscle-secreted destabilizing factors, such as Sema3A, on MN axons and motor function in ALS models.

Discussion

In this work, we demonstrated that the muscle toxicity in ALS is mediated by miR126-5p. We provided one specific mechanism for a well-described molecule (Sema3A), by which miR126-5p contributes to ALS pathology. We have also demonstrated that miR126-5p alterations facilitate axon degeneration and NMJ disruption in an ALS model as an outcome of presymptomatic elevations in the production and secretion of their target genes, which encode for destabilizing factors, such as Sema3A family members. Overexpressing miR126-5p in SOD1\textsuperscript{G93A} muscles inhibits the neurodegenerative process. These findings reveal how alterations in miR126-5p can be toxic to MNs, and identify a non-cell-autonomous neurodegeneration process in ALS (Fig. 6).

miR126-5p as a master regulator of proper NMJ function

Our results indicate that the expression of ALS-causative mutations results in the secretion of repellent factors, including a number of Type 3 Semaphorins and potentially other factors. It is thus likely that a general gene repression mechanism, specifically miR system, is altered under such conditions. This assumption is also consistent with the fact that miR alterations are apparent in various neurodegenerative diseases, including ALS (Haramati et al., 2010). Here we identified such an miR and showed how alterations in this specific miR can regulate the essential signaling pathways in MNs and can trigger neurodegeneration. Intriguingly, and in line with our findings, a very recent paper demonstrated a mechanism by which miR126-5p modulates Sema3A expression through SetD5 expression, and it emphasizes its positive effect on retinal endothelial cells’ survival (Villain et al., 2018). However, aside from targeting Sema3A and Sema3B, as well as NRP1 and NRP2, miR126-5p is predicted to regulate other Semaphorin signaling factors, such as Sema6D, PLXNA2, JNK2, JNK3, and PTEN. In addition, miR126-5p can regulate the ALS and motor-unit-related genes VEGF-A, SPAST, MMPs (Kaplan et al., 2014), AGRIN (Vilmont et al., 2016), and C9orf72, which are directly involved in ALS. Therefore, miR126-5p can serve as a master regulator of NMJ health by controlling multiple signaling pathways.

Sema3 alterations in ALS: settling the contradictory reports

A critical initiating event for the mechanism outlined above is the alterations in Sema3 signaling in ALS models and patients. Sema3A family members were previously found to be upregulated in terminal Schwann cells in the NMJs of SOD1\textsuperscript{G93A} mice (De Winter et al., 2006). Recently, Sema3A was also shown to be elevated in the motor cortex of postmortem ALS patients but not in their SC (Körner et al., 2016). Consistent with this, NRP1 antibodies, blocking the obligatory binding receptor for Sema3A, were injected into SOD1\textsuperscript{G93A} mice as a potential treatment (Venkova et al., 2014). However, anti-NRP1 blocking antibody had only a modest effect. Moreover, a recent study demonstrated that crossing mice expressing a truncated form of Sema3A with SOD1\textsuperscript{G93A} mice did not result in any rescue effect (Moloney et al., 2017). An explanation for a minor effect or not at all, as a result of blocking Sema3A activity, could be based on the idea of multiple toxic factors that play a role in ALS pathology. Another explanation for this contradiction could be the fact that Sema3A plays a more complex role in the biology of MNs. Indeed, Sema3A was shown to increase survival when added to mass cultures of mouse MNs (Molofsky et al., 2014) and human MNs (Birger et al., 2018). Consistent with this, deletion of the Sema3A gene specifically in spinal astrocytes resulted in a gradual loss of spinal MNs (Molofsky et al., 2014), thus suggesting that Sema3A has a positive effect when introduced near the cell body. When these findings are together with our results, apparently Sema3A has both positive and negative effects on MNs, perhaps depending on its specific subcellular localization. When Sema3A is secreted from muscles and targets distal axons at NMJs, it mediates their destabilization; however, when it is secreted by spinal astrocytes and targets MN soma, it acts as a survival factor. Thus, it is perhaps not surprising that a genetic approach to inhibit Sema3A in all cells in a mouse model of ALS had no effect in inhibiting the symptoms. The injection of anti-NRP1 may have been a bit more beneficial possibly either because it was able to inhibit Sema3A outside the CNS more effectively, or alternatively, because NRP1 blocks other Type 3 Semaphorins as well.
Autonomous versus non–cell-autonomous contributions to ALS progression

ALS is considered a complex disease, with unique MN features as well as non–cell-autonomous contributions (Ilieva et al., 2009; Musarò, 2013). Some evidence suggests that the NMJ is the first compartment to be disrupted in ALS rather than the MN soma; the disease is recognized as distal axonopathy in a non–cell-autonomous process (Fischer et al., 2004; Moloney et al., 2014). Two main cell populations that have been shown to play a role in distal axonopathy are glia and muscle cells, which secrete factors that influence MN survival and health (Moloney et al., 2014; Tsitkanou et al., 2016). However, the complexity of the disease and the involvement of several tissues raise controversies regarding the contribution of each tissue to the disease pathology. With skeletal muscle, few works have concluded that muscles do not play a role in ALS pathology. Reducing hSOD1G93A levels by injecting siRNA against its transcript directly into muscles of the SOD1G93A mouse model, as well as crossing Lox SOD1G93A with the Cre coding sequence under the control of the muscle creatine kinase promoter, or performing manipulations using Follistatin did not affect the disease onset and survival (Miller et al., 2006). In vitro application of muscle-CM from SOD1G93A-expressing muscle on healthy mass culture and ES cell-derived MNs resulted in no effect (Nagai et al., 2007). However, in contrast with these findings, evidence indicates that overexpressing mutant SOD1 protein specifically in healthy skeletal muscle induces an ALS phenotype and the degeneration of MNs, supporting a direct role for muscle in ALS physiology (Dobrowolny et al., 2008; Wong and Martin, 2010). Moreover, muscle from ALS patients and models has been shown to exhibit impaired mitochondrial function (Shi et al., 2010) and abnormalities in muscle biology (Manzano et al., 2012). Here, we demonstrated that applying presymptomatic SOD1G93A muscle-CM directly, and only on SOD1G93A-expressing MN axon tips, results in their degeneration, suggesting that both tissue types are necessary for exhibiting the disease phenotype. Furthermore, contradictory reports were published on transgenic mice expressing SOD1G93A only in the MNs. For example, Lino et al. (2002) showed a very mild phenotype, whereas Jaarsma et al. (2008) demonstrated a significant toxic effect. In this study, we showed that muscle-secreted factors are capable of modulating MN axons. Whereas wild-type muscle-secreted factors facilitate axon growth, several ALS-related mutations, expressed in muscles, result in the secretion of factors that cause retraction and degeneration when exclusively introduced to axons. At least one of these factors is Sema3A. The secretion of Sema3A by the muscle itself is likely to contribute to the instability of the MN axons. However, our results also show that ALS mutant muscles themselves cause axon degeneration and a delay in axon growth toward the muscles, but eventually the connections between axons and muscles are established. Thus, at least in our system, apparently the non–cell-autonomous contributions of the muscle are insufficient to recapitulate all the toxic effects on MNs. However, once the MNs also carry an ALS mutation, axons are more susceptible to degeneration by mutated muscle-CM (Fig. 3G,H), thus demonstrating the critical contribution of the MNs to ALS progression.

Do diseased muscles initiate axon degeneration or inhibit regeneration?

Our data suggest that muscles are involved in modulating MN health in ALS disease. We showed, both in vivo and in vitro, that muscles secrete destabilizing factors, such as Sema3A, as well as facilitate axon degeneration and NMJ disruption. Intriguingly, a previous study demonstrated that Sema3A expression is limited only to myosin IIb positive fibers, which are prone to be disrupted first in ALS (De Winter et al., 2006). These data support our findings in which the percentage of NMJs that express Sema3A and NRPI is reduced at the end stage of the disease, most likely along with the fast fatigable NMJs. However, although the suggested mechanism involves muscle-MN interaction, because of the nature of our experimental model, we cannot fully determine whether the mutated muscles act by initiating the degeneration of MN axons or by inhibiting their regrowth and NMJ repair, which was also suggested previously (Arbour et al., 2015). Perhaps muscle toxicity plays an active role in both pathways. However, future efforts should be made to resolve this issue.

miRs as a potential therapeutic strategy for ALS disease

In this paper, we demonstrated a positive effect of miR126-5p on ALS disease progression and suggested a potential therapeutic strategy for ALS disease. Nevertheless, our in vivo data show that the most significant positive effect of miR126-5p on ALS pathology was achieved at P90, whereas at later stages only modest effects were achieved. These results point to miR126-5p as a targeted treatment for an early phenotype but without a sustained beneficial contribution at later stages of ALS disease. However, keeping in mind that we injected miR126-5p into small parts of the whole GC muscle and only once at P60, as well as the fact that the efficiency of the procedure of the injection can also affect the efficacy of this treatment, we cannot rule out the possibility that a broader test of long-term efficacy will result in a stronger and more positive outcome. An alternative future study should address this issue by crossing a conditional tissue-specific knock-out of miR126-5p mice with SOD1G93A.

Considering that ALS is a multifactorial disease, and that miRs are predicted to regulate a wide range of metabolic and signaling pathways, manipulating their subcellular levels in neurons, muscles, or glia, miRs should generally be explored as a potential therapeutic strategy or tool for treatment of ALS and possibly other neurodegenerative diseases.

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Maimon et al. • miR126-5p Dysregulation in ALS Physiology

5492 • J. Neurosci., June 13, 2018 • 38(24):5478–5494

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