Cytoplasmic pool of U1 spliceosome protein SNRNP70 shapes the axonal transcriptome and regulates motor connectivity

Graphical abstract

Highlights

- Spliceosome protein SNRNP70 localizes within axonal RNA granules
- SNRNP70 is required in motor neurons for axonal growth and synaptogenesis
- Strictly cytoplasmic SNRNP70 can drive key aspects of motor connectivity
- Cytoplasmic SNRNP70 modulates the local transcriptional landscape

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In brief

mRNA splicing factors are unexpectedly found in neurites, where their functions are poorly understood. Nikolaou et al. show that U1 spliceosome protein SNRNP70 associates with mRNAs in motor axons and regulates neuronal connectivity. At the molecular level, it modulates local transcriptome composition and mRNA trafficking.

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Cytoplasmic pool of U1 spliceosome protein SNRNP70 shapes the axonal transcriptome and regulates motor connectivity

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SUMMARY
Regulation of pre-mRNA splicing and polyadenylation plays a profound role in neurons by diversifying the proteome and modulating gene expression in response to physiological cues. Although most of the pre-mRNA processing is thought to occur in the nucleus, numerous splicing regulators are also found in neurites. Here, we show that U1-70K/SNRNP70, a component of the major spliceosome, localizes in RNA-associated granules in zebrafish axons. We identify the extra-nuclear SNRNP70 as an important regulator of motor axonal growth, nerve-dependent acetylcholine receptor (AChR) clustering, and neuromuscular synaptogenesis. This cytoplasmic pool has a protective role for a limited number of transcripts regulating their abundance and trafficking inside axons. Moreover, non-nuclear SNRNP70 regulates splice variants of transcripts such as agrin, thereby controlling synapse formation. Our results point to an unexpected, yet essential, function of non-nuclear SNRNP70 in axonal development, indicating a role of spliceosome proteins in cytoplasmic RNA metabolism during neuronal connectivity.

INTRODUCTION
RNA processing, transport, and local translation play a central part in the normal development and function of neural circuits. These processes are extensively regulated by numerous RNA-binding proteins (RBPs) interacting with RNAs inside and outside the nucleus.1,2 In recent years, much progress has been made in understanding molecular mechanisms required for normal RNA metabolism and transport.3–5 Conversely, defects in RBP expression, localization, and function have been linked to numerous neurodevelopmental and neurodegenerative diseases including autism, schizophrenia, bipolar disorders, spinal muscular atrophy, and amyotrophic lateral sclerosis.6–9 Many RBPs participate in the regulation of pre-mRNA splicing in the nucleus, but some of these splicing regulators are also found in the neurites of developing and mature neurons. For instance, the neuronal splicing factor Nova co-localizes with its target RNAs in the dendrite.10 Moreover, components of the major, U1/U2-dependent spliceosome have been found to localize in the developing axonal growth cone.11 The splicing protein SFPQ, which is enriched in the nucleus, additionally localizes to mouse dorsal root ganglion axons where it regulates RNA granule co-assembly and trafficking of mRNAs essential for axonal viability such as LaminB2 and Bclw.12 We showed that non-nuclear SFPQ is also required for normal maturation of zebrafish motor axons.13 Notably, another RBP better known for its nuclear functions, U1 small nuclear ribonucleoprotein 70K (SNRNP70), is present in zebrafish motor axons at readily detectable levels,13 although the functional significance of this finding has not been investigated.

SNRNP70 associates with the U1 small nuclear RNA (U1 snRNA) and, together with other subunits, forms the U1 snRNP component of the major spliceosome. SNRNP70 contains a tail domain mediating binding with the Sm proteins, an α helix domain, an RNA recognition motif (RRM) forming contacts with the U1 snRNA, and two low complexity arginine/serine domains involved in interactions with SR proteins.14,15 SNRNP70 is required for both constitutive and alternative pre-mRNA splicing.16 Its function outside the nucleus is currently unknown.

Here, we show that SNRNP70 localizes to cytoplasmic puncta closely associated with RNA granules within axonal compartments. We demonstrate that the cytoplasmic pool of SNRNP70 is an important regulator of motor axonal growth, nerve-dependent acetylcholine receptor (AChR) clustering, and neuromuscular synaptogenesis. Transcriptomics and functional assays further indicate that extra-nuclear SNRNP70 regulates cytoplasmic mRNA processing. Together, our study identifies a novel function of a major spliceosome protein in neurites, modulating the abundance and motility of transcripts and regulating the production of alternative splice variants during neuronal circuit formation.
RESULTS

SNRNP70 co-localizes with RNAs in developing axons

We examined SNRNP70 distribution in zebrafish using an antibody raised against a highly conserved region of its human homolog. In line with the key role of this protein in pre-mRNA splicing, we detected robust nuclear staining across embryonic and larval tissues including GFP-labeled motor neurons (MNs) in Tg(mnx1:GFP) animals (Figure S1A). As reported previously, SNRNP70 was also observed inside motor axons innervating the muscles (Figure S1A) and was often found near neuromuscular junctions (NMJs), large synapses between MNs, and muscle fibers expressing AChRs on the post-synaptic side (Figure S1C).

To further analyze the subcellular localization of SNRNP70, we dissociated and cultured neurons derived from Tg(mnx1:GFP) zebrafish embryos at 28 h post fertilization (hpf). At day 1 in vitro, GFP+ neurons had visible growth cones extending from the soma and in some cases formed simple axonal arbors (Figure 1A). Immunostaining showed that SNRNP70 protein localized to axons of these GFP+ neurons in the form of small puncta (Figures 1A and 1B).

Several RBPs are known to associate with ribonucleoprotein (RNP) complexes transporting RNA molecules along axons and dendrites. To find out if the cytoplasmic fraction of SNRNP70 could participate in this process, we imaged a functionally active enhanced green fluorescent protein (eGFP)-tagged version of human SNRNP70 (hSNRNP70-eGFP) and labeled newly synthesized RNAs with a fluorescent derivative of uridine-5'-triphosphate (Cy5-UTP) to visualize fluorescent Cy5-RNA+ RNP granules in axons. Confirming our immunofluorescence data above, mosaic expression of the recombinant hSNRNP70-eGFP protein showed robust nuclear localization in brain and spinal cord (Figure 1C). We also observed multiple eGFP+ puncta co-localizing with RNAs in neuronal processes. Some of these puncta appeared to move stochastically, whereas others showed either slow or fast unidirectional movements along the neurite (Figure 1C; Video S1). These results suggest that SNRNP70 is present in axons where it associates and moves along with RNAs.

SNRNP70 is cell-autonomously required in MNs for neuromuscular assembly

To understand how SNRNP70 might contribute to MN development, we first generated a complete loss-of-function line targeting the first coding exon of the zebrafish snrnp70 gene (exon2) using CRISPR-Cas9. DNA sequencing of target-specific PCR products confirmed that the mutant snrnp70 allele (kg163) carried a deletion of 13 bases (Figure S2A). The deletion resulted in a frameshift immediately after amino acid 16 and a truncation 66 amino acids after the mutation, thus expected to disrupt all known functional domains of the SNRNP70 protein (Figures S2B and S2C). In addition to truncating the open reading frame, the mutant allele led to a reduced snrnp70 transcript expression (data not shown), likely due to nonsense-mediated decay (NMD) targeting mRNA with a premature translation termination codon. Homozygous mutants for snrnp70 (snrnp70−/− thereafter referred to as null) were obtained from heterozygotes and larval tissues including GFP-labeled motor neurons (MNs) and larval tissues including GFP-labeled motor neurons (MNs) in Tg(mnx1:GFP) animals (Figure S1A). As reported previously, SNRNP70 was also observed inside motor axons innervating the muscles (Figure S1A) and was often found near neuromuscular junctions (NMJs), large synapses between MNs, and muscle fibers expressing AChRs on the post-synaptic side (Figure S1C).

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SNRNP70 immunofluorescence across different cell types, including MNs (Figure S1A), and a significantly reduced number of fluorescent puncta colocalizing with motor nerves as compared with the wild-type (WT) control (Figures S1A and S1B). Due to maternal RNA contribution, snrnp70-null embryos were not affected until the mid-somite stage (data not shown). From this stage onward, we observed morphological defects including abnormal tail extension, heart edema, and cell death. Brain regions were particularly sensitive to the loss of snrnp70 indicating a critical role of SNRNP70 in neural development (data not shown). Despite the dramatic defects seen in the brain, the spinal cord, and somites were much less affected between 18 and 28 hpf allowing us to examine the role of SNRNP70 in the formation of the NMJ, a synapse established early in neural circuit development. In zebrafish, there are four subtypes of primary MNs and several subtypes of secondary MNs. Primary MN growth cones exit the spinal cord around 18 hpf and reach the most ventral part of the myotome by 28 hpf. Using the Tg(mnx1:GFP) line, we examined how the absence of the SNRNP70 protein affected normal MN development. The gross morphology of null MNs was similar to siblings at 28 hpf, except for a reduction in the total length and thickness of the motor nerves (Figures 2A–2C). At 48 hpf, motor axonal development was severely disrupted—several aberrant branches, reduced branching within the myotome, and limited innervation of the lateral myosepta were observed (Figures S2D–S2F).

One of the earliest hallmarks of the NMJ assembly is the clustering of AChRs on the surface of muscle cells adjacent to the muscle-innervating axons, replacing the initial aneural AChR clustering. To investigate how neuromuscular assembly may be affected in snrnp70-null embryos, we stained pre-synaptic MN terminals with an SV2-specific antibody and AChR-containing post-synaptic structures on muscle fibers with a fluorescently tagged α-bungarotoxin (α-BTX). At 28 hpf, there were visible clusters of AChRs apposed to the SV2+ pre-synaptic sites in siblings. However, in null animals, the AChR clusters were reduced, whereas the SV2+ structures appeared normal (Figure 2D). Consistent with this phenotype, there was a significant decrease in the degree of co-localization between SV2 and α-BTX within the myotome (Figure 2E). Decreased AChR clustering was also evident at 48 hpf, a stage of active neuromuscular assembly (Figures S2G and S2H). The lack of post-synaptic AChRs is unlikely due to the abnormal development of the myotome. Indeed, somitic muscle differentiation occurs normally in the null embryos, with typical V-shaped somitic boundaries, proper differentiation of both slow and fast muscle fibers, and the formation of sarcomeres (Figures S3A and S3B). The absence of AChR clusters on muscle fibers, without initial defects in muscle differentiation, suggests that the primary defect is in the MNs.

To directly assess whether SNRNP70 is required cell-autonomously in MNs for the induction of AChRs at NMJs, we performed cell mosaic experiments. Cell transplantation from Tg(mnx1:GFP) donors into the spinal neural plate territory of WT hosts at 70% epiboly resulted in clones of 1–5 fluorescently marked donor MNs. Sibling MNs transplanted into WT embryos produced axonal projections fully integrated into the host environment. Quantification of appositions formed between the sibling transplanted neurons (GFP+/SV2+ puncta) and the WT host
Figure 1. SNRNP70 localizes to axons

(A) A representative image of a dissociated motor neuron at 1 day in vitro derived from Tg(mnx1:GFP) animals at 28 hpf. The sample was stained with anti-SNRNP70 to reveal the localization of the protein in neurites and DAPI to mark nuclei. Scale bars, 10 μm.

(B) Inset showing a higher magnification of the motor axon in (A). Arrows indicate SNRNP70+ puncta within the GFP+ axon. Scale bars, 5 μm.

(C) Time-lapse of neurons at 48 hpf sparsely labeled with hSNRNP70-eGFP. Top: images show GFP-labeled neurons co-expressing UTP-Cy5. Bottom: insets depict axon segments showing association between Cy5-RNA granules (magenta) and hSNRNP70-eGFP (green) signals (arrows indicate the same punctum during the imaging session with time shown in the bottom right corner of individual time points). In the single-channel images, the hSNRNP70-eGFP fluorescence signal has been outlined and overlayed in the UTP-Cy5 signal for a direct comparison. Representative examples from three independent experiments. Scale bars, 5 μm.

See also Figure S1 and Video S1.
muscle fibers (α-BTX+ puncta) showed that there was a good amount of overlap between pre- and post-synaptic markers at the NMJs (Figures 3A and 3D, with red arrows in Figure 3A indicating appositions). Transplantations of null MNs into WT hosts resulted in ventral projections and myotome innervations at 48 hpf; however, the overlap of pre- and post-synaptic markers was variable (Figures 3B and 3C with red and cyan arrows indicating the presence or absence of appositions, respectively). To investigate the underlying reasons, we examined the innervation sites of the transplanted neurons and found that null MNs terminating in areas of the myotome with sparse or no WT motor axons in their proximity had fewer appositions compared with null MNs innervating motor-axon-dense regions of the myotome (Figure 3D). The compensation mechanism from the host MNs supporting connectivity of transplanted null MNs is likely causing the bimodal behavior of mutant axons. We next transplanted WT

Figure 2. Loss of SNRNP70 affects motor neuron growth and neuromuscular connectivity

(A) Representative maximum intensity projection images of Tg(mx1:GFP) sibling and null trunk, lateral views with anterior to the left, at 28 hpf showing motor nerves innervating the myotome. Scale bars, 50 μm.

(B and C) Quantifications showing the length and thickness of motor nerves in the two groups. All graphs show mean values ± SEM. ****p < 0.0001; ***p < 0.001, two-tailed unpaired t test, n = 10 animals per group in two independent experiments.

(D) Maximum intensity projection images of sibling and null embryos at 28 hpf stained with anti-SV2 antibody to mark the pre-synaptic locations and a fluorescently tagged α-BTX to label AChRs on post-synaptic structures. Images are lateral views with anterior to the left. Scale bars, 25 μm.

(E) Quantification of the degree of overlap between SV2 and α-BTX. The graph shows mean values ± SEM. ****p < 0.0001, two-tailed unpaired t test, n = 10 animals per group in two independent experiments

See also Figure S2.
Figure 3. SNRNP70 is cell-autonomously required in motor neurons for neuromuscular assembly

(A) A representative image showing transplanted sibling motor neurons derived from a Tg(mnx1:GFP) line innervating a WT host myotome that has been stained with anti-SV2 and $\alpha$-BTX to mark pre- and post-synaptic sites, respectively. Bottom: inset depicting the apposition (red arrows) of pre- (within the GFP$^+$ motor neurons) and post-synaptic sites within a small region of the myotome. Scale bars, 25 $\mu$m.

(B and C) Examples of transplanted GFP$^+$ null motor neurons innervating WT host myotomes that have been stained with anti-SV2 and $\alpha$-BTX. In (B), null GFP$^+$ motor axons terminate in a motor-axon-sparse region of the myotome, whereas in (C), they terminate in a motor-axon-dense region. Bottom: insets depicting the apposition (red arrows) of pre- (within the GFP$^+$ motor neurons) and post-synaptic sites regions of the myotome. Cyan arrows indicate the absence of host AChR clusters adjacent to transplanted GFP$^+$/SV2$^+$ terminals. Scale bars, 25 $\mu$m.

(D) Quantification of the degree of overlap between SV2 and $\alpha$-BTX in the three groups of transplanted motor axons. The graph shows mean values ± SEM. ***p< 0.001, one-way ANOVA, n = 6 donor/host pairs for sib-to-WT group, n = 3 donor/host pairs for null-to-WT group in three independent experiments. See also Figure S3.

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A  

|       | 48 hpf |       | 48 hpf |       |
|-------|--------|-------|--------|-------|
| sib   |        | sib/cyt-hSNRP70 |        | null   |
| (1)   |        | (2)    |        | (3)    |
| null/cyt-hSNRP70 |        |        |        | (4)    |
|       | 48 hpf |

B  

|       | 28 hpf |       | 28 hpf |       |
|-------|--------|-------|--------|-------|
| sib   |        | sib/cyt-hSNRP70 |        | null   |
| (1)   |        | (2)    |        | (3)    |
| null/cyt-hSNRP70 |        |        |        | (4)    |
|       | 28 hpf |

C  

Touch evoked response score

- sib
- sib/cyt-hSNRP70
- null/cyt-hSNRP70

D  

α-BTX/SV2 overlap (Pearson's r)

- sib
- sib/cyt-hSNRP70
- null/cyt-hSNRP70

E  

Motor nerve length (μm)

- sib
- sib/cyt-hSNRP70
- null/cyt-hSNRP70

(legend on next page)
cells into the mutant environment and found that these WT MNs although shorter in the null environment, matured normally, and established numerous NMJs (Figures S3C and S3D). By contrast, the mutant MNs were unable to establish NMJs in this null environment (Figures S3C and S3D). These observations indicate that SNRNP70 is required cell-autonomously in MNs for the clustering of AChR at the NMJ and that this effect is modulated by the density of WT motor axons surrounding the null donor axon terminals.

The cytoplasmic pool of SNRNP70 regulates neuromuscular connectivity

We wondered if the newly identified role of SNRNP70 in NMJ development depended on the cytoplasmic fraction of this RBP. To this end, we rescued the loss of the endogenous SNRNP70 protein in the null background using its either WT or cytoplasmic-only transgenic variants. The WT transgene, Tg(UAS:hSNRNP70-eGFP), encoded a full-length hSNRNP70-eGFP fusion under the control of the upstream activation sequence (UAS) promoter. Crossing Tg(UAS:hSNRNP70-eGFP) fish to the inducible Tg(ubi:ERT2-Gal4) line allows ubiquitous overexpression of hSNRNP70-eGFP upon the addition of 4-hydroxytamoxifen (4-OHT) (Figures S4A and S4C). We analyzed four groups of embryos obtained from heterozygote Tg(UAS:hSNRNP70-eGFP) line produced a similar restoration of neuromuscular connectivity by regulating aspects of motor axonal growth and synaptogenesis. Tg(ubi:ERT2-Gal4) line. Embryos derived from the same clutch were sorted into four groups according to genotype: (1) sib, (2) sib/cytsib, (3) null, and (4) null/cytsib. Representative images of live embryos at 48 hpf from three independent experiments. Scale bars, 25 μm.

Cytoplasmic SNRNP70 modulates transcript abundance

To examine the transcriptome-wide effects of SNRNP70, we analyzed 28 hpf sibling and null embryos either positive or negative for cytosolic hSNRNP70-eGFP by RNA sequencing (RNA-seq). A principal component analysis of the gene expression data showed clear segregation between sibling and null animals regardless of the expression of cytosolic hSNRNP70-eGFP (Figure S6A). We first evaluated gene expression changes across all annotated genes in zebrafish and discovered 2,015 genes upregulated and 2,498 genes downregulated following the loss of SNRNP70 (Figure S6B; Table S1).

To assess the possible contribution of cytoplasmic SNRNP70 to this large-scale gene regulation program, we compared the...
Figure 5. Cytoplasmic SNRNP70 regulates transcript abundance and motile behavior
(A and B) Data are based on RNA-seq in 28 hpf embryos derived from the ubiquitous overexpression of cytosolic hSNRNP70-eGFP in sibling and null animals using the Tg(ubi:ERT2-Gal4;UAS:hSNRNP70D NLS3xNES-eGFP) line. Embryos were sorted into four groups according to genotype: (1) sib, (2) sib/cyt-hSNRNP70, (3) null, and (4) null/cyt-hSNRNP70. (C) Representative images of rab1bb-Cy5 expression at 32 hpf. (D) Summary data for the number of normal rab1bb puncta/10μm. (E) Brightness of normal rab1bb puncta. (F) Fraction of motile normal rab1bb puncta. (legend continued on next page)
null/cyt-hSNRNP70 with the null group. We found 347 rescued transcript events whose expression was either increased or decreased in null animals and significantly restored in the null/cyt-hSNRNP70 group (from a total of 1,009 genes whose expression changed significantly between these two groups (Figures S6C and S6D, Table S1). Gene ontology (GO) enrichment analysis of these 347 transcript levels showed association to structural constituents of the ribosome as well as protein targeting to the endoplasmic reticulum (ER) and plasma membrane affecting nervous system development (Figure S6E). Further examination of these 347 transcript rescue events showed that there was a stronger enrichment for genes downregulated in null animals and upregulated by cytoplasmic SNRN70 (Figures 5A and 5B). This suggested that cytoplasmic SNRN70 might associate with at least some of these transcripts to regulate their abundance outside the nucleus.

Given that many of the rescued transcripts play important roles in nervous system processes, we decided to investigate whether changes in the cytoplasmic expression of SNRN70 were sufficient to modulate the abundance of these transcripts in axons. We chose rab1bb as a case study for being a component of mRNA granules present in the synaptic transcriptome and subject to local translation. Mosaic blastomere injections of fluorescently labeled rab1bb-Cy5 together with a mCherry-caax RNA resulted in the appearance of fluorescent axonal rab1bb+ RNA puncta within sparse mCherry-labeled neurons (Figure S7A), suggesting that rab1bb RNA is present within axonal RNA granules under physiological conditions. Using time-lapse imaging, we investigated the behavior and dynamics of axonal rab1bb+ puncta (Figure 5C). In animals deficient for SNRN70 (null), the number and fluorescence intensity of rab1bb+ puncta were significantly reduced (Figures 5D and 5E). Moreover, the motility of rab1bb+ puncta was compromised with very few motile puncta observed compared with sib (Figure 5F). The expression of cytosolic hSNRN70-eGFP in nulls rescued the brightness and number of rab1bb+ puncta, as well as the ratio of motile over the total number of puncta (Figures 5C-5F; Video S2).

To better understand the decrease in the brightness and number of rab1bb+ puncta found in null axons, we quantified rab1bb+ puncta in the soma and found that although soma number does not significantly change between the different genetic backgrounds, the brightness of soma rab1bb+ as well as the ratio of rab1bb+ puncta moving from the soma to the axon is reduced in the null and rescued by cytosolic hSNRN70-eGFP (Figures S7B-S7E; Video S3). These findings suggest that the decreased number of rab1bb+ puncta found in null axons partially results from defective trafficking. The reduction in the relative brightness of rab1bb+ puncta could either be due to a decrease in the number of rab1bb-Cy5 molecules packaged into granules or a decreased stability and/or increased degradation of rab1bb-Cy5 transcripts in the absence of cytoplasmic SNRN70.

The presence of rab1bb+ RNA puncta in axons and the regulation of its abundance and trafficking by cytoplasmic SNRN70 indicates that SNRN70 co-localizes with rab1bb mRNA in axonal RNA granules. Indeed, mosaic neuronal expression of hSNRN70-eGFP and rab1bb-Cy5 mRNA shows that the majority of SNRN70+ puncta are colocalizing with rab1bb mRNA in both soma and axons (79%). A small number of rab1bb+ puncta does not overlap with SNRN70 protein (19%), and very few SNRN70+ puncta have no rab1bb mRNA (7%) (Figures 5G and 5H). Taken together, these data suggest that cytoplasmic SNRN70 localizes to mRNA granules and regulates transcript abundance and axonal transport.

**Cytoplasmic SNRN70 modulates alternative splicing**

Given that SNRN70 is an essential splicing factor, we also examined changes in pre-mRNA splicing patterns (Figure 6A). The loss of SNRN70 (comparing null with the sib group) resulted in significant alternative splicing changes with most altered events to be either cassette exon (CE) splicing or intron retention (IR) in null animals. Changes in alternative 3' (Alt3) and 5' (Alt5) splice site usage were also observed (Figure 6B; Table S2). In the case of CE, the majority of altered events resulted in shorter alternatively spliced isoforms (i.e., increased exon skipping), whereas, in the case of IR, there was an equal number of increases and decreases in IR resulting in longer and shorter isoforms, respectively (Figure 6B).

Surprisingly, the transgenic expression of cytosolic hSNRN70-eGFP in nulls recovered a fraction of affected alternatively spliced events (comparing null/cyt-hSNRN70 with the null group, 93 events in total), suggesting a complex SNRN70 cytoplasmic function. In these 93 rescued events,
A

Longer AS

Alternative 5' (Alt5)

Alternative 3' (Alt3)

Intron retention (IR)

Cassette exon (CE)

Shorter AS

B

Number of AS events (MV.dPSI > 0.1)

null vs sib

C

Number of AS events (MV.dPSI > 0.1)

Non-rescued

Rescued

D

Rescued events
(null/cyt-hSNRNP70 vs null)

E

chr10: 2,847,500 2,849,500 2,851,500

F

cchr25: 31,010,160 31,010,500

G

rapgef5a exon 7

H

ptprnb exon 16

I

syt8 intron 4

J

myo10 intron 17

(legend on next page)
CE and IR were also the most abundant categories (Figure 6C; Table S2). The majority of CE-restored events were a correction of defective exon skipping found in null animals, whereas the majority of IR rescues was the decrease of null abnormal IR (Figure 6D). Schematic plots for representative examples of CE and IR events are highlighted in Figures 6E and 6F, respectively.

GO enrichment analysis of the rescued alternatively spliced events showed a significant enrichment for synaptic vesicle recycling proteins, protein tyrosine phosphatase signaling molecules, and vesicle tethering complexes (Figure S7F), i.e., essential components of axonal growth and synapse function. Four alternatively spliced events were chosen for validation based on the following criteria: (1) the splicing event was affected by cytoplasmic SNRNP70, (2) the gene has a basal expression value of ≥2 cRPKM (corrected reads per kilobase of target transcript sequence per million of total reads) in sibling control animals, and (3) the gene encoding the affected transcript has a role in neuron development/function. Cytoplasmic SNRNP70-dependent CE events were validated by semi-quantitative RT-PCR (rapgef5a exon 7 and ptpnb exon 16). Supporting the RNA-seq results, we found exon skipping to be significantly increased in null, and its splicing was significantly recovered in null/cyt-hSNRNP70 (Figures 6G and 6H). We also validated IR events by RT-qPCR (syt8 intron 4 and myo10 intron 17), showing a significant increase in null and a significantly restored normal splicing in null/cyt-hSNRNP70 (Figures 6I and 6J). These rescued events were unlikely to be due to altered expression of nuclear splicing factors as the expression of all spliceosome proteins (except for SNRNP70) either remained unchanged or increased in null animals and showed no response to the expression of cytoplasmic SNRNP70 (Table S3). Taken together, the data indicate that the cytoplasmic fraction of SNRNP70 modulates the relative abundance of alternatively spliced transcripts associated with neuronal development and synaptic function.

The cytoplasmic fraction of SNRNP70 controls the production of Z+AGRN isoforms

We wondered whether the effect of cytoplasmic SNRNP70 on the splice form composition could explain the partial rescue of neuromuscular connectivity in null animals. Agrin (AGRN) is a multidomain proteoglycan secreted by the MN growth cone, which binds to LRP4/Musk receptor complex on the muscle fiber plasma membrane. The activation of this receptor complex leads to a signal transduction pathway that induces the neural clustering of AChRs.30–33 It has been previously shown that the ability of AGRN to induce the clustering of AChR depends in part on alternative splicing to produce Z+AGRN isoforms that have an insertion of either or both of two micro-exons encoding eight (z8 micro-exon) or eleven (z11 micro-exon) amino acids, respectively.34–37

Therefore, we set out to examine whether the alternative splicing of Z+AGRN isoforms is impaired in null embryos, and if so, whether this was rescued by cytosolic hSNRNP70-eGFP. Since the annotation of the zebrafish genome is currently incomplete, these exons are absent from the database of alternative events. Through sequence alignment with mouse and human, we mapped the two putative micro-exons (named z8 and z11) between exons 33 and 34 of the zebrafish AGRN locus. Using RT-PCR, three alternatively spliced z+agn variants were identified, containing insertions of either eight (33-28-34 allele), eleven (33-21-34 allele), or nineteen amino acids (33-z8-21-34 allele), respectively. Moreover, a comparison of the amino acid sequence of the Z+AGRN domain encoded by the two micro-exons showed sequence conservation between humans and zebrafish (Figure 7A). We found that while the total transcript expression of non-z+agn (agn 33–34 (z2)) isoforms increases in the null (Figure 7B), the expression of the three z+agn transcripts is reduced following the loss of SNRNP70 (Figures 7C–7E). Expression of cytosolic hSNRNP70-eGFP brought the expression levels of non-z+agn isoforms back to normal and rescued the expression of two out of three z+agn isoforms (33-28-34 and 33-z11-34) (Figures 7B–7E). These results indicate that (1) the cytoplasmic pool of SNRNP70 regulates the normal production of Z+AGRN isoforms and (2) this regulation involves reciprocal changes in fractional abundance of z+agn and non-z+agn splice variants.

The RT-PCR analysis above suggests that changes in the expression of z+agn isoforms may partly underpin the ability of cytoplasmic SNRNP70 to control neuromuscular connectivity, thus placing Z+AGRN downstream of cytoplasmic SNRNP70. To test the ability of Z+AGRN to induce AChR clusters in the absence
Figure 7. Cytoplasmic SNRNP70 regulates the abundance of Z+AGRN splice variants

(A) Agrin Z+ (Z+AGRN) isoforms identified by RT-PCR are shown schematically, with conserved residues between humans and zebrafish shown in red. Arrows depict the position of primers used for the qPCR analysis in (B)–(E).

(legend continued on next page)
of SNRNP70, we exogenously provided Z+AGRN. As expected, mock-treated null animals had very few AChR clusters in comparison with mock-treated sib controls (Figures 7F and 7G). Ectopic Z+AGRN increased the number of AChR clusters in both sib and null animals compared with the mock-injected control groups (Figures 7F and 7G). We found that although the number of AChR clusters in nulls is recovered following Z+AGRN application (Figure 7G), the average size of these clusters remains similar to mock-injected nulls (Figure 7H). The data indicate that although Z+AGRN acts downstream of cytoplasmic SNRNP70 to initiate the clustering of AChRs, the inability of ectopic Z+AGRN to restore proper clustering size indicates that SNRNP70 is also acting downstream and/or independently of Z+AGRN.

**DISCUSSION**

The wiring of the nervous system is a complex process that depends on the availability of specific mRNAs and proteins at the right time (e.g., during axon guidance or synapse formation) and in the right place (e.g., growth cone and synaptic sites). To date, many axonal and dendritic RBPs have been found to play essential roles during brain connectivity48–50 although the molecular mechanisms carrying these roles are still obscure. Our study shows that the major spliceosome U1 protein SNRNP70 is closely associated with mRNA molecules within normal neurites. We show that the major spliceosome U1 protein SNRNP70 is sufficient to partially restore the expression of a cytoplasmic form of SNRNP70. Indeed, motor neuronal expression of the cytoplasmic-only SNRNP70 is sufficient to partially restore motor axonal growth and neuromuscular synaptogenesis defects in snrnp70 null animals. These findings indicate that the non-nuclear pool of this spliceosome protein regulates essential aspects of neuromuscular connectivity. Our cytoplasmic expression experiments rescue null motor phenotypes partly through an SNRNP70-mediated modulation of cytoplasmic pre-mRNA processing, supporting a similar role for the endogenous axonal pool of SNRNP70.

Precise recognition of the 5’ splice site by the U1 snRNP particle is essential for both constitutive and alternative pre-mRNA splicing, with the latter being subjected to further regulation.41,42 A previous study has found that U1C, another U1 snRNP-associated protein, is an important mediator of CE splicing in zebrafish.43 This study was interpreted to reflect nuclear function of U1C. Although our snrnp70 loss-of-function data further confirm the role of U1C particle components in alternative splicing regulation during development, our findings give insights into the roles of spliceosome components outside the nucleus. It is widely understood that the U1 snRNP assembly initiates within the cytoplasm before SNRNP70 (independently of U1 snRNA and the Sm core of the particle) is transported to the nucleus.25,34–36 Our study provides the first evidence that the cytoplasmic pool of a spliceosome component is functionally active independently from its nuclear translocation. One possibility is that SNRNP70 may be involved in a form of extra-nuclear immature-mRNA processing. Tissue-specific alternative splicing is a powerful source of protein diversity. This mechanism is particularly important in the nervous system where isoform variation is involved in cell recognition, synapse formation, synaptic plasticity, ion channel function, and neurotransmission.47,48 Although post-transcriptional regulation commonly occurs in the nucleus, recent studies indicate that spliceosome components and splicing factors are found in axons and dendrites where they retain their potential to promote pre-mRNA splicing.47,49,50 This suggests that SNRNP70 could be interacting with other splicing factors to promote the complete splicing of partially spliced pre-mRNAs found in neurites. For instance, Nova1 and Nova2, two well-conserved neuron-specific splicing factors, regulate the alternative splicing of z+agrm isoforms and are essential for Z+AGRN-mediated AChR cluster formation at the NMJ.51

Novo proteins were also found to regulate brain- and subtype-specific alternative splicing programs required for axon guidance and neural circuit formation.52,53 Like SNRNP70, Nova proteins were also found to localize in neuromuscular junctions.10 Therefore, it will be interesting to see whether extra-nuclear SNRNP70 and Nova proteins interact in the context of axon guidance and motor connectivity. The possibility for local alternative splicing is an attractive idea in the context of neurons. Many of the neuronal populations controlling our behaviors have the nucleus tens of centimeters away from the site of active synapses. The option of local splicing would be an advantageous mechanism to generate alternative transcripts known to be essential for the rapid modulation of synaptic connectivity and other neuronal functions. Although we did not observe any change in known splicing factor expression in our RNA-seq datasets, we cannot completely rule out the possibility that cytoplasmic SNRNP70 might be regulating transcripts encoding a splicing factor, which in turn influences nuclear alternative splicing.

Our studies also point to a role of SNRNP70 in modulating the abundance and motility of mRNA transcripts outside the nucleus. Such transcripts will need to be assembled into cytoplasmic RNP complexes, which are membrane-less organelles involved in the transport of RNA molecules along neurites. The presence of two C-terminal low complexity domains in the

**[B-E] RT-qPCR analysis in sibling and null animals as well as following ubiquitous overexpression of cytosolic hSNRNP70-eGFP using Tg(ubi:ERT2-Gal4; UAS:hSNRNP70::JNL::NES-eGFP) embryos at 28 hpf. Primers were designed to specifically amplify the expression of non-z+agrm (agrm 33-34 (L2) isoforms (B), as well as the three z+agrm isoforms: 33-z8-34 (C), 33-z71-34 (D), and 33-z8-271-34 (E). The upper graph shows mean values ± SEM for fold change in expression in relation to control animals (sib). ***p < 0.0001; **p < 0.001; *p < 0.01; **p < 0.05; ns, not significant, one-way ANOVA, datapoints represent two independent biological replicates per group.**

**[(F)]** Representative images depicting a superficial layer of the trunk myotomes showing the distribution of x-BTX-labeled AChR clusters in mock- and Z+AGRN-treated sibling and null animals at 48 hpf. Images are lateral views with anterior to the left. Scale bars, 50 μm.

**[(G)]** Quantification of the number of x-BTX clusters in the four groups. The graph shows mean values ± SEM. ****p < 0.0001; **p < 0.01; *p < 0.05; ns, not significant, one-way ANOVA, n = 21 animals per group in two independent experiments.

**[(H)]** Graph showing mean values ± SEM of the average size of x-BTX clusters in the four groups. ****p < 0.0001; **p < 0.05; ns, not significant, one-way ANOVA, n = 21 animals per group in two independent experiments.**
SNRNP70 protein was shown to facilitate its phase separation into membrane-less RNA granules, which supports the presence of a functional SNRNP70 granule in axons. Although very little is known about the composition of this granule, one potential explanation for the downregulation of transcripts levels in SNRNP70 deficient animals is the absence of cytoplasmic SNRNP70 from these granules, which will indirectly prevent such transcripts from targeting the axon and lead to their degradation. In the case of SFPQ, it was shown to be required for the co-assembly of an RNA regulon essential for axonal viability. Similarly, SNRNP70 could be involved in packing a specific set of mRNA targets into axonal RNA granules. However, our cytoplasmic SNRNP70 rescue experiment, showing that the motility of rab1bb transcripts is restored, suggests that SNRNP70 could also be required for the transport of RNA granules. This could be achieved through a direct interaction with mRNA transcripts, mediated through its RRM motif, like what has been previously shown for Staufen in dendrites.

In conclusion, our studies reveal unexpected cytoplasmic functions of a key spliceosome factor, SNRNP70, during the development of neuronal connections. Further work will be needed to examine the full extent of the biological functions of SNRNP70 in neurites and the mechanisms by which it controls the cytoplasmic transcriptome composition and influences the establishment and maintenance of neuronal connectivity.

**STAR★METHODS**

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**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2022.10.048.

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**AUTHOR CONTRIBUTIONS**

C.H. conceived the research project and secured funding. N.N., P.M.G., F.H., R.T., E.V.M., and C.H. designed the experiments. N.N. analyzed the data. F.H. performed the bioinformatic analysis of RNA-seq data. N.N. wrote the manuscript with input from all authors.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Chick anti-GFP      | ThermoFisher Scientific | Cat# PA19533; RRID: AB_1074893 |
| Rabbit anti-GFP     | Abcam  | Cat# ab6556; Lot: GR3351259; RRID: AB_305564 |
| Rabbit anti-SNRNP70 | Sigma  | Cat# AV40276; Lot: QC9623; RRID: AB_1857332 |
| Mouse anti-SV2      | DSHB   | Cat# 5V2; RRID: AB_2315387 |
| Mouse anti-F59      | DSHB   | Cat# F59; RRID: AB_528373 |
| Mouse anti-F310     | DSHB   | Cat#F310; RRID: AB_531863 |
| Mouse anti-Histone-H3 | Cell Signaling Technology | Cat# 96C10; RRID: AB_1642229 |
| Mouse anti-α Tubulin | Cell Signaling Technology | Cat# DM1A; RRID: AB_1666201 |
| Goat anti-mouse IgG (H+L) Alexa Fluor® 488 | ThermoFisher Scientific | Cat# A11001 |
| Goat anti-rabbit IgG (H+L) Alexa Fluor® 488 | ThermoFisher Scientific | Cat# A11008 |
| Goat anti-mouse IgG (H+L) Alexa Fluor® 568 | ThermoFisher Scientific | Cat# A11004 |
| Goat anti-rabbit IgG (H+L) Alexa Fluor® 568 | ThermoFisher Scientific | Cat# A21069 |
| Goat anti-mouse IgG (H+L) Alexa Fluor® 633 | ThermoFisher Scientific | Cat# A21050 |
| Goat anti-rabbit IgG (H+L) Alexa Fluor® 633 | ThermoFisher Scientific | Cat# A21070 |
| Goat anti-rabbit HRP | Abcam  | Cat# ab205718 |
| Goat anti-mouse HPR  | Abcam  | Cat# ab205719 |
| Chemicals, peptides, and recombinant proteins |        |            |
| Recombinant human BDNF | Sigma | Cat# B3795 |
| Recombinant Z+AGRN | R&D Biosystems | Cat# 550AG |
| Penicillin-Streptomycin | Sigma | Cat# P4333 |
| Gentamicin          | Gibco  | Cat# 15750060 |
| Granzyme B inhibitor | Calbiochem | Cat# 368056 |
| α-Bungarotoxin-Alexa Fluor® 555 | ThermoFisher Scientific | Cat# B35451 |
| Pronase             | Sigma  | Cat# 10165921001 |
| DNase               | QIAGEN | Cat# 79254 |
| DAPI                | Sigma  | Cat# D8417 |
| HOECHST             | ThermoFisher Scientific | Cat# H3569 |
| UTP-Cy5             | Perkin Elmer | Cat# NEL583001EA |
| 4-Hydroxytamoxifen (4-OHT) | Abcam | Cat# ab141943 |
| Tricaine (MS-222)   | Sigma  | Cat# A5040 |
| Leibovitz L-15 medium | Gibco | Cat# 11415049 |
| N-2 Supplement      | Gibco  | Cat# 17502048 |
| MACS NeuroBrew-21 Supplement | Miltenyi-Biotec | Cat# 130-093-566 |
| FluorSave Reagent   | Millipore | Cat# 345789 |
| Low melting point agarose | ThermoFisher Scientific | Cat# 16520050 |
| cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail | Roche | Cat# 11836170001 |
| PhosSTOP (Phosphatase inhibitors) | Roche | Cat# 04906845001 |
| NuPAGE™ LDS Sample Buffer | ThermoFisher Scientific | Cat# NP0007 |
| NuPAGE™ Sample Reducing Agent | ThermoFisher Scientific | Cat# NP0009 |
| 4-12% Bis-Tris protein gels | ThermoFisher Scientific | Cat# NW04120BOX |
| SeeBlue™ Plus2 Pre-stained Protein Standard | ThermoFisher Scientific | Cat# LC5925 |
| Pico PLUS Chemiluminescent Substrate | ThermoFisher Scientific | Cat# 34577 |

(Continued on next page)
### Critical commercial assays

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| T7 RiboMAX RNA Production kit | Promega | Cat# P1320 |
| SP6 mMessage mMachine kit | ThermoFisher Scientific | Cat# AM1340 |
| QuickChange II Site-Directed Mutagenesis kit | Agilent | Cat# 200523 |
| RNase Mini kit | QIAGEN | Cat# 74104 |
| TruSeq Stranded Total RNA Library Prep Gold kit | Illumina | Cat# 20020598 |
| TruSeq RNA UD Indexes | Illumina | Cat# 20022371 |
| Agilent Technologies 2200 TapeStation | Agilent | Cat# 50675582 |
| D1000 ScreenTape assay | Agilent | Cat# 50675583 |
| Kapa Library quantification kit | Roche | Cat# KK4824 |
| NovaSeq XP chemistry workflow | Illumina | Cat# 20021664 |
| NovaSeq S1 reagent kit | Illumina | Cat# 20027465 |
| First strand cDNA synthesis kit | ThermoFisher Scientific | Cat# K1612 |
| DreamTag Green PCR Master mix | ThermoFisher Scientific | Cat# K1081 |
| LightCycler 480 SYBR Green I Master kit | Roche | Cat# 04707516001 |

### Deposited data

| RNA-seq of snrnp70 mutant zebrafish and following rescue by overexpression of cytoplasmic SNRNP70. | ArrayExpress | Accession code: E-MTAB-12301 |

### Experimental models: Organisms/strains

| AB wild type strain of zebrafish | European Zebrafish ResourceCenter (EZRC) | N/A |
| Tg(mx1:GFP)^iso7g | Flanagan-Stee et al. | N/A |
| Tg(lubi:ERT2-Gal4)^iso10T9 | Gerety et al. | N/A |
| Tg(mx1:Gal4)^iso20T9 | Zelenchuk et al. | N/A |
| snrnp70^iso163 | This paper | N/A |
| Tg(UAS:hSNRNP70-eGFP)^iso22T9 | This paper | N/A |
| Tg(UAS:hSNRNP70.dNLS3xNES-eGFP)^iso25T9 | This paper | N/A |

### Oligonucleotides

See Table S4

### Recombinant DNA

| pDR274 | Hwang et al. | N/A |
| pCS2-Cas9 | Gagnon et al. | N/A |
| pN2-5UAS:eGFP | Fredj et al. | N/A |
| pT2-5UAS:MCS:eGFP | This paper | N/A |
| pminiTol2 | Balciunas et al. | N/A |
| pN1-CMV:hSNRNP70-eGFP | Huranová et al. | N/A |
| pT2-5UAS:hSNRNP70-eGFP | This paper | N/A |
| pT2-5UAS:hSNRNP70.dNLS3xNES-eGFP | This paper | N/A |
| pCS-TP | Kawakami et al. | N/A |
| HuC:Gal4 | Walker et al. | N/A |
| pCS-CMV:H2B-mRFP1 | Megason | N/A |
| pCS2^-mCherry-caax | Gordon et al. | N/A |

### Software and algorithms

| Fiji | Schindelin et al. | https://imagej.net/software/fiji/ |
| GraphPad Prism 9.3.1 | GraphPad Software Company | https://www.graphpad.com/scientific-software/prism/ |
| ZenBlue3.3 software | Carl Zeiss Company | https://www.zeiss.com/microscopy |

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RESOURCE AVAILABILITY

Lead contact
Further information and request for resources should be directed to and will be fulfilled by the lead contact, Professor Corinne Houart (corinne.houart@kcl.ac.uk).

Materials availability
Unique reagents generated in this study are available from the lead contact upon request.

Data and code availability
RNA sequencing datasets generated by this study have been deposited on ArrayExpress. The accession number for the snrnp70 and rescue datasets is E-MTAB-12301.

This paper does not report original code.

Any additional information required to re-analyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Source of zebrafish used in this study are reported below and in the key resources table.

Animals
Zebrafish were reared at 28.5 °C on a 14 hr light/10 hr dark cycle. Embryos produced by natural crosses were raised in Danieau’s solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM Ca(NO3)2, 5.0 mM HEPES, pH 7.2). We used the following existing transgenic lines and strains: Tg(mnx1:GFP)ml2Tg, Tg(ubi:ERT2-Gal4)nim10Tg, Tg(mnx1:Gal4), and AB. The following lines were newly generated for the purpose of this study: snrnp70kg163, Tg(UAS:hSNRNP70-eGFP)kg323Tg, and Tg(UAS:hSNRNP70 D NLS3xNES- eGFP)kg325Tg. This work was approved by the local Animal Welfare and Ethical Review Bodies (King’s College London and University of Bath) and was carried out in accordance with the Animals (Scientific Procedures) Act, 1986, under project licenses (P70880F4C and PP8698401) from the United Kingdom Home Office.

METHOD DETAILS

Generation of snrnp70 knock-out allele
The kg163 null allele of snrnp70 was generated by injecting the one-cell stage AB embryos with 250 pg Cas9 mRNA and 40 pg sgRNA. The sgRNA was generated by inserting a guide sequence (5’-GAACGGTATGGGGTCCCGC-3’) into vector pDR274 (Addgene) and transcribing using the T7 Ribomax RNA Production kit. Cas9 was transcribed from the plasmid pCS2-Cas9 (Addgene) using the SP6 mRNASeq mRNAMachine kit. Adult F0 animals were outcrossed with AB zebrafish and F1 offspring embryos were screened for CRISPR-induced ‘indel’ mutations by high melt resolution (HRM) analysis using forward (5’-TCTCTTC CAGGTGAACAATGAC-3’) and reverse (5’-TCCCAGTTGAGGCAGGAACG-3’) primers (Table S4). Embryos with shifted HRM curves compared to un-injected AB embryos were sequenced using forward (5’-CTTAACCTTGCATGAGGTGCTCC-3’) and reverse (5’-AGGAGCAATTCCGCAGTAGG-3’) genotyping primers (Table S4) to reveal the type of mutation. This led to the identification of an F0 founder that produces embryos with a 13bp deletion and was used to generate the stable line used in this study.

Generation of transgenic constructs
Sequences for all primers used are listed in Table S4. The pT2-5UAS:MCS-eGFP construct was generated by digesting pN2-5UAS-S:eGFP (gift from Martin Meyer, King’s College London, UK) with AseI and AflII restriction endonucleases. The 1.4 kb product containing five repeats of the Upstream Activation Sequence (5UAS) and a hsp70 minimal promoter, a multiple cloning site, the enhanced green fluorescent protein (eGFP) sequence and an SV40 polyA was blunted at both ends using a Klenow fragment of DNA
polymerase I and cloned into the EcoRV site of the pminiTol2 plasmid. To generate the pT2-5UAS:hSNRNP70-eGFP construct, a 1.4 kb fragment containing the hSNRNP70 sequence was isolated from the pN1-CMV:hSNRNP70-eGFP plasmid (gift from David Stanek, Institute of Molecular Genetics, Czech Republic) by digestion with Nhel and BamHI restriction endonucleases. The fragment was then directionally cloned into the Nhel/BamHI sites downstream of the UAS motifs of the pT2-5UAS:mCS-GFP construct generating C-terminally fused enhanced green fluorescent protein (hSNRNP70-eGFP). To generate a cytosolic hSNRNP70 construct, the pT2-5UAS:hSNRNP70-eGFP plasmid was used as a template for sequential site directed mutagenesis using QuickChange II kit to mutate the two nuclear localization signals (NLS) of SNRNP70 protein as previously described. For mutating the PCR product using the SP6 mMessage mMachine kit. To enable the fluorescent visualization of the NLS, we used a forward primer: 5'-CCCGCTCTGCAGAAAAAGTTGGAAGAGCTTGAGC-3' and a reverse primer: 5'- CAAGGACACCTCTGCAATCAACGGGAAGTCTCGTGTGTTTGAAGGGGAG-3' to modify the amino-acid sequence of 166-GKK-168 to 166-DFP-168. For NLS2, we used a forward primer: 5'- GAGGCTGAGAGGGGCGGAAACCGTGAAGGCCCAATCTAGTCTGGTGAGGAGGGCTGCTGGTGTTACAGAA-3' and a reverse primer: 5'- TTCTGATCCACGCCAGGCCCTCTCCTCGTTAGTGCTAAGGCTCAGTTCAGCTCAACGGCGTCGCCCTCAGCTCAACGGCGTCC-3' to alter the amino-acid sequence of 186-GWKPRR-191 to 186-ANLVDL-191. To introduce three copies of MEKK nuclear export signals (NES); amino acid sequence: ALQKKEELLEL at the C-terminal end of hSNRNP70 protein, we used two rounds of restriction free cloning. In the first round, we used a forward primer: 5'- GATCGACGGCGTCGCCACCATGGCTCTGCAGAAAAATGTTGAAGGCTTG-3' and a reverse primer: 5'- CTCTTCCAACTTTTTCTGCAGAGCCATCTCATCCAGCTCAAGCTCTTCCAACTTTTTCTGC-3' encoding the NES sequence were used to insert a copy of NES. For the second round, a new forward primer: 5'-CCGCGCCGGTGCACCCCGC- CACCGCTCTGCAAGAAATGTTGAAGGCTTGAGCGAGGCTCAGTTCAGCTCAACGGCGTCGCCCTCAGCTCAACGGCGTCC-3' and a reverse primer: 5'- CTCTTCCAACTTTTTCTGCAGAGCCATCTCATCCAGCTCAAGCTCTTCCAACTTTTTCTGC-3' were used. Following the second round of restriction free cloning, we identified clones that contained 2x as well as 3x copies of NES. We thus decided to use the latter one (pT2-5UAS:hSNRNP70-JNL5xNES-eGFP) for all of our cytoplasmic SNRNP70 rescue experiments. All constructs generated were confirmed by nucleotide sequencing.

DNA microinjections and generation of transgenic lines
Fertilized zebrafish AB embryos were microinjected with 1.8 nl of an injection solution containing 15-25 ng/μl DNA and 17 ng/μl Tol2 transposase RNA. Tol2 RNA was transcribed from the plasmid pCS-TP35 (gift from Koichi Kawakami, National Institute of Genetics, Japan) using the SP6 mMessage mMachine kit. For establishing stable transgenic lines, animals injected with either pT2-5UAS:hSNRNP70-eGFP or pT2-5UAS:huSNRNP70-JNL5xNES-eGFP DNA constructs were raised to adulthood. Adult F0 animals were outcrossed with Tg(ubi:ERT2-Gal4) zebrafish and F1 offspring were screened for germline transmission of the transgene.

Time-lapse imaging of axonal SNRNP70
The pT2-5UAS:hSNRNP70-eGFP effector plasmid was co-injected with an activator plasmid containing Gal4 driven by an upstream HuC pan-neuronal promoter (HuC:Gal4)53 (gift from Martin Meyer, King’s College London, UK) together with 100 μM UTP-Cy5 inside a single blastomere at 4-8 cell stage. Embryos at 48 hpf were mounted in 1% low melting point agarose (Sigma) in Danieau’s solution. Imaging was performed using a Zeiss LSM 880 Fast Airyscan confocal microscope equipped with GaAsP spectral detectors and a 20x/1.0 NA water-immersion objective (Carl Zeiss). Excitation was provided using 488 nm (for GFP) and 633 nm (for Cy5) solid state lasers. Images were captured at 0.2 Hz at a 0.06 x 0.06 μm resolution (512 x 512 pixels) and 1 A.U. pinhole aperture and then processed for Airyscan imaging.

Single cell analysis of cytosolic hSNRNP70-eGFP distribution
The pT2-5UAS:hSNRNP70-JNL5xNES-eGFP effector construct was co-injected with the HuC:Gal4 plasmid together with a previously published pCS-CMV:H2B-mRFP+p65 (Addgene) inside the fertilized egg. Injected embryos were mounted in 1% low melting point agarose in Danieau’s solution. Imaging was performed using a Zeiss Axio Observer.Z1 LSM 880 confocal microscope equipped with GaAsP spectral detectors and a 40x/1.1 NA water-immersion objective (Carl Zeiss). Excitation was provided using 488 nm (for GFP) and 561 nm (for RFP) solid state lasers. High resolution images were captured at 0.1 x 0.1 μm resolution (512 x 512 pixels) and 1 A.U. pinhole aperture and subsequently processed for Airyscan imaging.

Imaging of axonal rab1bb-Cy5 puncta
The entire coding region of rab1bb gene together with endogenous 5’ and 3’ UTRs was amplified from a wild-type cDNA library using forward (5’- ATTATAGTACACTATAAGAGAGGAAGAGGCTTGAGACACAC-3’) and reverse (5’- AAACCCTCAGGAA GACGCT-3’) primers. The forward primer contains an SP6 polymerase binding site. Capped RNA was synthesised directly from the PCR product using the SP6 mMessage mMachine kit. To enable the fluorescent visualization of rab1bb* puncta, 1 μl of UTP-Cy5 was added to the reaction. For mCherry-caax RNA, the pCS2*“mCherry-caax” plasmid (gift from Kristen Kwan, University of Utah, USA) was linearised with NotI and capped RNA was then synthesized using the SP6 mMessage mMachine kit. 80pg of rab1bb-Cy5 RNA and 20pg of mCherry-caax RNA were co-injected in a single blastomere at 32- to 64-cell stage. Embryos at 32 hpf were mounted in 1% low melting point agarose in Danieau’s solution. Imaging was performed using a Zeiss Axio Observer.Z1 LSM 880 confocal microscope equipped with GaAsP spectral detectors and a 40x/1.1 NA water-immersion objective (Carl Zeiss). Excitation was provided using 488 nm (for eGFP) 561 nm (for mCherry) and 633 nm (for Cy5) solid state lasers. Images were captured
Z+AGRN (0.3 ng) diluted in 0.1% bovine serum albumin was injected into fertilised eggs derived from heterozygote snrnp70 null zebrafish embryos. 53 A recombinant Z+AGRN protein that contains a nine amino acid insert at the Z site was used to treat snrnp70 null zebrafish embryos. Z+AGRN (0.3 ng) diluted in 0.1% bovine serum albumin was injected into fertilised eggs derived from heterozygote snrnp70 null zebrafish embryos. As a mock control, bovine serum albumin was injected into a separate group of eggs. Embryos at 48 hpf were fixed and incubated with 1:100 rabbit1bb+ solution for the rest of the experiment. A recombinant Z+AGRN protein that contains a nine amino acid insert at the Z site was used to treat snrnp70 null zebrafish embryos. For measuring the ratio of motile over the total number of puncta, a motile punctum was defined as one that appeared to move at least once during the entire duration of the movie.

Z+AGRN treatment

A recombinant Z+AGRN protein that contains a nine amino acid insert at the Z site was used to treat snrnp70 null zebrafish embryos. Z+AGRN (0.3 ng) diluted in 0.1% bovine serum albumin was injected into fertilised eggs derived from heterozygote snrnp70 null zebrafish embryos. As a mock control, bovine serum albumin was injected into a separate group of eggs. Embryos at 48 hpf were fixed and incubated with 1:100 α-Bungarotoxin (α-BTX)-555 conjugate at 4°C overnight. Samples were imaged using a Zeiss Axio Observer.Z1 LSM 880 confocal microscope equipped with GaAsP spectral detectors. Excitation was provided using a 561 solid state laser. α-BTX was imaged using a 40x/1.3 NA oil objective (Carl Zeiss) at a 0.41 x 0.41 μm resolution (512 x 512 pixels) and 1 A.U with 0.75 μm sectioning z-interval. For quantifying the number and size of α-BTX clusters, a single confocal section in the superficial part of the myotome was used. Using Fiji, the image was thresholded and the analyze_particles plugin was then used to identify and count the number of clusters.

4-OHT treatment for GAL4 induction

4-Hydroxytamoxifen (4-OHT) was dissolved in ethanol at a final stock concentration of 50 mM and stored in the dark at −20°C. To induce Gal4 activity in Gal4ERT2-expressing offspring, embryos at sphere stage (~ 4 hpf) were washed in a petri dish containing Danieau’s solution, all medium was removed and replaced with fresh Danieau’s solution containing 2 μM of 4-OHT. The treated embryos were immediately put into a dark 28.5°C incubator to allow for efficient induction and remained in 4-OHT-containing Danieau’s solution for the rest of the experiment.

Motor neuron mosaic experiment

Cell transplantations were done as previously described. 13 Briefly, offspring derived from heterozygote snrnp70+/-:Tg(mnx1:GFP) crosses were used in this study. Around 20 dorso-posterior epiblast cells collected from late gastrula stage donor embryos (80%-100% epiboly) were placed at the equivalent location in the same stage wild-type host embryo. The donor embryos were identified as siblings or nulls by PCR at 28 hpf. Embryos that led to the incorporation of the donor cells in the ventral spinal cord and GFP-positive motor axons were selected for further analysis.

Tissue preparation and cryosectioning for histology

Zebrafish embryos and larvae (48 - 120 hpf) were euthanized with 4 mg/mL Tricaine (f) and immersion fixed overnight at 4°C in 4% paraformaldehyde. Fixed animals were embedded in an agar-sucrose solution (1.5% agar, 5% sucrose) in PBS and let to set. The agar blocks were taken out of their moulds, placed into 30% sucrose solution. Following an overnight incubation at 4°C, blocks are frozen by gradual immersion into liquid nitrogen and stored at −80°C until sectioning. Transverse sections of 20 μm thickness were cut using a Bright Instruments-OTF5000 cryostat and subsequently stored at −80°C until further use.

Immunohistochemistry

For whole mount immunohistochemistry, 4% paraformaldehyde-fixed embryos were washed with PBS, permeabilized with 0.25% Trypsin in PBS for 20 minutes and blocked with 10% goat serum/PBS at room temperature (RT) for 1 hour. Embryos were incubated with chick anti-GFP 1:500, anti-SNRNP70 1:100, anti-SV2 1:200, anti-F59 1:10, anti-F310 1:10 diluted in 10% goat serum/PBST (1% Triton X-100) at 4°C overnight, washed with PBST (1% Triton X-100) and incubated with Alexa Fluor 488/568/633 secondary antibodies (1:500) diluted in 10% goat serum/PBST (1% Triton X-100) at 4°C overnight. For immunohistochemistry experiments where anti-SNRPNP70 antibody was used, an antigen retrieval step was incorporated in the protocol prior to the permeabilization step where samples were incubated with 150mM Tris-HCl (pH 9.0) for 5 minutes at room temperature followed by 15 minutes at 70°C. For staining Acetyl Choline receptors (AChRs), we incubated embryos with 1:100 α-Bungarotoxin (α-BTX)-555 conjugate at 4°C overnight. In some cases, HOECHST or DAPI were used at 1:1000 to stain cell nuclei. Cryosections were rehydrated in PBS for 10 minutes and then blocked in for 1 hour before incubating primary antibodies at 4°C overnight. Sections were then washed and incubated with secondary antibodies for 2 hours at room temperature.

Imaging of immunofluorescent samples

Following immunohistochemistry, samples were imaged using a Zeiss Axio Imager.Z2 LSM 800 confocal microscope equipped with 2x GaAsP spectral detectors. Excitation was provided using 405, 488, 561 and 633 nm solid state lasers. Whole mount immunostainings of slow and fast muscle fibers using F59 and F310 antibodies, respectively, were imaged using a 20x/0.8 NA air objective (Carl Zeiss) at a 0.62 x 0.62 μm resolution (512 x 512 pixels) and 1 A.U with 1 μm sectioning z-interval. Whole mount labeling of pre- and posy-synaptic NMJ markers using anti-SV2 and α-BTX was imaged using a 40x/1.3 NA oil objective (Carl Zeiss) at a 0.31 x 0.31 μm resolution (512 x 512 pixels) and 1 A.U with 0.75 μm sectioning z-interval. Immunostainings of all other whole mount
samples as well as cryosections were imaged using a 40x/1.3 NA oil objective (Carl Zeiss) at a 0.31 x 0.31 μm resolution (512 x 512 pixels) and 1 A.U. High magnification images of cryosections showing spinal cord and peripheral nerves were captured at a 0.08 x 0.08 μm resolution (512 x 512 pixels) and 1 A.U. pinhole aperture.

Quantification of motor nerve SNRNP70+ puncta on cryosections
For quantifying the number of SNRNP70+ puncta inside motor nerves, a single plane confocal section was used. Using Fiji, the GFP signal in motor nerves was thresholded to create a binary image. A mask derived from this binary image was transferred into the SNRNP70 immunostaining, and the analyze_particles plugin was then used to identify and count SNRNP70+ puncta within the GFP-labeled motor nerve. Due to the high fluorescent background caused by the antibodies, many large puncta can still be seen, even in the absence of SNRNP70. To avoid incorporating such puncta into our analysis, we limited the size of puncta to less than 0.2μm². The number of puncta was then normalised to the area of the motor nerve.

Co-localization analysis of pre- and post-synaptic NMJ markers
NMJ analysis was done as previously described. Briefly, following confocal imaging of anti-SV2 and α-BTX within somites 10 and 11, datasets were opened in Fiji. Anti-SV2 and α-BTX channels were merged and a maximum projection of a z-stack covering a depth of 15 μm was made. Only the ventral part of the myotomes was used for this analysis. Channels were split and background was subtracted using a rolling ball radius of 10 pixels. Finally, the Pearson’s r was calculated using the coloc2 plugin. For NMJ analysis of transplanted MNs, the GFP signal was thresholded to create a binary image, which was then used as a mask for identifying the degree of overlap between anti-SV2 and α-BTX in the GFP+ transplanted MNs.

Zebrafish primary cell culture
Embryos of the Tg(mnx1:GFP) line were collected, incubated overnight in Danieau’s solution containing 0.01% methylene blue, Penicillin-Streptomycin and 50 μg/mL Gentamicin and bleached at 28 hpf using 0.0026% sodium hypochlorite. Bleached embryos were then dechorionated using pronase, transferred into a tube containing 1 mL of cell dissociation solution (25% Cell Dissoaciation Buffer (enzyme free), 3.25x10⁻⁸ M EDTA, PBS, pH 8.0) and stored on ice. Embryos were then dissociated using a series of autoclaved Fisherbrand Pasteur pipets with cotton plugs with successively smaller bore diameters. After approximately 15 minutes, when chunks of embryos were no longer visible, the dissociated cells were passed through a Corning 40 μm cell strainer into a 50 mL centrifuge tube. Extra cell dissociation solution was used to rinse the cell strainer membrane, pushing through any material stuck on top. The tube was centrifuged at 300g for 7 minutes at 4°C. Cell dissociation solution was removed from above the cellular pellet, before resuspension in Zebrafish Neural Medium (ZNM) consisting of Leibovitz L-15 medium, 1X N-2 Supplement, 1X MACS NeuroBrew-21 Supplement, 10ng/mL BDNF, 2% FBS, 1X Penicillin-Streptomycin & 50 μg/mL Gentamicin. Cell solution was subsequently divided, plating onto Corning Biocoat Poly-D-Lysine/Laminin-coated coverslips in a 24-well-plate. The plate was sealed by wrapping with parafilm and left overnight at RT. Cells were fixed at one day in vitro (DIV1). ZNM was removed from each well before rinsing once with PBS. 1 mL of 4% PFA was added to each well for 30 minutes at RT. Following removal of PFA, coverslips were washed 2 X 5-minutes with PBST (1% Triton X-100). Coverslips were then incubated with blocking solution (PBST and 5% Goat Serum) for 1 hour at RT. This was removed and coverslips were incubated with fresh blocking solution containing chick anti-GFP (1:1000 dilution) and anti-SNRNP70 (1:200 dilution) overnight at 4°C. This solution was removed, and coverslips subsequently underwent 3X PBST washes at RT whilst on a shaker. They then had further blocking solution added to them containing Alexa Fluor 488/568 secondary antibodies in addition to DAPI (1:2000 dilution). Coverslips were incubated at RT for 2 hours. After removing the solution from coverslips, they underwent 3 X PBST washes over the course of a few hours. Coverslips were mounted onto glass slides with 10μl FluorSave Reagent. Samples were imaged using a Zeiss Axio Imager.Z2 LSM 800 confocal microscope equipped with GaAsP spectral detectors and a 40x/1.3 NA oil objective (Carl Zeiss). Excitation was 405 nm (for DAPI), 488 nm (for GFP) and 561 nm (for SNRNP70). Images were captured at a 0.2 x 0.2 μm resolution (512 x 512 pixels) and 1 A.U. pinhole aperture and were then analyzed using Fiji.

Morphological analysis of motor nerves
Live Tg(mnx1:GFP) embryos were mounted in 1% low melting point agarose in Danieau’s solution. Imaging was performed using a Zeiss Axio Examiner.Z1 LSM 800 confocal microscope equipped with GaAsP spectral detectors and a 20x/1.0 NA water-immersion objective (Carl Zeiss). Excitation was provided using a 488 nm solid state laser. Image stacks of motor nerves were captured at a 0.62 x 0.62 μm resolution (512 x 512 pixels) and 1 A.U. pinhole aperture with 0.5 μm sectioning z-interval. Datasets were opened in Fiji and motor nerves in somites 9-12 were traced using the simple_neurite_tracer plugin. Motor nerve length was measured from the spinal cord exit point to the most distal end of the nerve. The length of myosepta innervation was measured from the most ventral part of the motor nerve until the distal end of the nerve near the horizontal myoseptum and normalised to the total distance to the horizontal myoseptum to get the percentage of myosepta length innervated. Motor nerve thickness was quantified by making maximum intensity projections and using a straight line to measure the thickness of the nerve at the level of the choice point on the horizontal myoseptum.
Assessment of startle response

Touch-evoked startle response was measured as previously described. Briefly, startle responses were elicited by gently touching 48 hpf animals using a blunt tool and measured using a scale from 0 to 3 as follow: 0, no movement; 1, flicker of movement but no swimming; 2, movement away from probe but with impaired swimming; and 3, normal swimming.

Nucleocytoplasmic fractionation

Between 30-50 embryos at 30 hpf stage were dechorionated and transferred into a tube. Water was removed and 100 µl of lysis buffer (10 mM HEPES; pH 7.5, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 1% NP-40, 5 µg/mL Granzyme B inhibitor with protease and phosphatase inhibitor cocktails) was added. Tubes were placed on ice for 15 minutes with intermittent mixing. To separate cells from yolk proteins, samples were centrifuged at 300 xg (2,000 rpm) at 4°C for 5 minutes to pellet the cells. The cell pellet was resuspended in 1000 µl of fresh lysis buffer. Tubes were vortexed to disrupt cell membranes and then centrifuged at 12,000 xg (13,000 rpm) at 4°C for 10 minutes. The supernatant was stored at -70°C till further use as cytoplasmic extract. The pelleted nuclei were washed gently twice with the cell lysis buffer and resuspended in 50 µl of nuclear extraction buffer (20 mM HEPES; pH 7.5, 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 5 µg/mL Granzyme B inhibitor with protease and phosphatase inhibitor cocktails) and incubated on ice for 30 minutes. Nuclear extract was collected by centrifugation at 12,000 xg (13,000 rpm) at 4°C for 15 minutes, and either used immediately or stored at -70°C until further use.

Western blotting

For western blotting, 26 µl of either nuclear or cytoplasmic fractions were mixed with 10 µl of LDS sample buffer and 4 µl of reducing agent and then loaded into 4-12% Bis-Tris protein gels together with a pre-stained protein standard. Gels were transferred into PVDF membranes and immunostained using rabbit anti-GFP (1:500 dilution), anti-Histone H3 (1:1000 dilution), and anti-alpha-tubulin (1:5000 dilution). For the detection of primary antibodies, goat anti-rabbit (1:5000 dilution) and goat anti-mouse (1:5000 dilution) HRP-conjugated secondary antibodies were used, and protein detection was carried out using a Pico PLUS Chemiluminescent Substrate. Membranes were scanned using the Vilber Fusion SL imager.

RNA sequencing

Embryos derived from an outcross of snrnp70+/−; Tg(ubi:ERT2-Gal4); Tg(UAS:hSNRNP70 ΔNLS3xNES-eGFP) with snrnp70+/−; Tg(ubi:ERT2-Gal4) animals were treated with 2 µM 4-OH (as described above). Treated embryos were screened for GFP fluorescence and also sorted into siblings and nulls (based on the brain morphogenesis phenotype) at 24 hpf to produce the following four experimental groups: sib, sib/cyt-hSNRNP70, null and null/cyt-hSNRNP70. Total RNA from whole embryos was isolated from these four pools at 28 hpf using a RNeasy Mini kit and eluted in nuclelease-free water. Total RNA samples (100-500 ng) were hybridized with Ribo-Zero Gold to substantially deplete cytoplasmic and mitochondrial RNA from the samples. Stranded RNA sequencing libraries were prepared as described using the TruSeq Stranded Total RNA Library Prep Gold kit with TruSeq RNA UD Indexes. Purified libraries were qualified on an Agilent Technologies 2200 TapeStation using a D1000 ScreenTape assay. The molarity of adapter-modified molecules was defined by quantitative PCR using the Kapa Biosystems Kapa Library Quant kit. Individual libraries were normalized to 1.30 nM in preparation for Illumina sequence analysis. Sequencing libraries (1.3 nM) were chemically denatured and applied to an Illumina NovaSeq flow cell using the NovaSeq XP chemistry workflow. Following transfer of the flowcell to an Illumina NovaSeq instrument, sequencing was performed using a NovaSeq S1 reagent kit to obtain 50 nucleotide paired-end reads. All library preparation and sequencing steps were carried out by the Huntsman Cancer Institute High-Throughput Genomics facility, University of Utah, USA.

Bioinformatics

For gene expression analyses and splicing analyses of 28 hpf RNA sequencing data, reads were aligned to zebrafish VASTDB library using VAST-TOOLS alignment tool and gene read counts output was enabled. Alignment was done as follows:

```
vast -- tools align fwd.fastq.gz rev.fastq.gz -- sp Dre -- cores 40 -- expr
```

Splicing data from each sample were combined using VAST-TOOLS' combine tool to obtain an INCLUSION table. Differential splicing analysis between samples was then determined using VAST-TOOLS’ “diff” command and a minimum change in Percent Spliced In (dPSI) value of 0.05 was requested. This was done as follows:

```
vast -- tools diff -- i INCLUSION.tab -- a sampleA -- b sampleB -- noPDF -- m 0.05 -- c 40 -- d output.txt
```

Differential gene expression analysis was carried out using edgeR package with the estimateGLMRobustDisp function. Downstream processing of splicing and gene expression data were done in R using custom scripts.

GO enrichment analysis

GO term analysis of the affected groups of genes was performed using the Gene Ontology Enrichment resource. We identified significantly enriched terms (P < 0.05) using the Panther overrepresentation test for enrichment with a Fisher’s exact test and used the following databases: GO/Biological processes, GO/Cellular component and GO/Molecular function.

Assessment of startle response

Touch-evoked startle response was measured as previously described. Briefly, startle responses were elicited by gently touching 48 hpf animals using a blunt tool and measured using a scale from 0 to 3 as follow: 0, no movement; 1, flicker of movement but no swimming; 2, movement away from probe but with impaired swimming; and 3, normal swimming.
**Quantitative and semi-quantitative RT-PCR**

Total RNA was extracted as described above for RNA sequencing. cDNA was synthesized using a First strand cDNA synthesis kit after treating the isolated RNA with DNaseI. Semi-quantitative RT-PCR reactions were performed using DreamTaq Green PCR Master mix on an Eppendorf thermocycler. We used the following primers (also listed in Table S4): rapgef5a_exon6 forward 5'-GACGAC TATTCTCCACACCA-3', rapgef5a_exon8 reverse 5'-GTCGTCTTCACATCTGTCCA-3', ptprnb_exon15 forward 5'- ACCATTCC AGAGTGAAAGCTG-3', ptprnb_exon17 reverse 5'- AGCCCTTCATCGGCCAGTAG-3'. RT-qPCR reactions were performed using LightCycler 480 SYBR Green I Master kit on a Roche LightCycler 96 system and data analyzed using the LightCycler96 software. To obtain relative fold changes, transcript expression levels were first normalized to an endogenous control gene *actin-b1*, and then normalised again to the total expression of the gene in sibling control animals. We used the following primers: *myo10_intron17* forward 5'-GTAACTGTTCCTCCAGTAT-3', *myo10_exon18* reverse 5'-CAGGCCGTCACCAGCTCAAA-3', *myo10_exon18* forward 5'-TTTGAAGCAGTAGAAGCCCTG-3', *myo10_exon19* reverse 5'-GTACTAAGGTTGACACCTG-3', *syt8_exon4* forward 5'- CCTTAA CCCCCGTGTCAATG-3', *syt8_intron4* reverse 5'- TAGCATTGTCACAAGGTAA-3', *syt8_exon5* forward 5'- AAGGAGCTAACC GAGTGAC-3', *syt8_exon5* reverse 5'- GAGATCTCTCCACTTTCA-3', *agrin_exon33+34* forward 5'-TCACCAAGCGAGAA AGCC-3', *agrin_exonZ8+34* forward 5'-AGATCCCGAGCGAGAAAGCC-3', *agrin_exon33+Z11* forward 5'-TCACCAAGCGCCCT GAGTCA-3', *agrin_exonZ8+Z11* forward 5'-AGATCCCGAGCGCTTCGCAATA-3', *agrin_exon45* reverse 5'-CAAACGCGCAGCTA-3', *actin-b1* forward 5'-TTACACCACCGCGGAAAGA-3', *actin-b1* reverse 5'-CTGACCAGCGACCTCATA-3'.

**Identification of z+agrn micro-exons**

Through sequence alignment of zebrafish *agrn* gene with mouse and human z+agrn isoforms we mapped the two putative micro-exons between exons 33 and 34 of the zebrafish AGRN locus. We performed RT-PCR using a forward primer in exon 33 (5'- AC ACGGAGCGCATGTGTTA-3') and a reverse primer in exon 34 (5'- CAACTCCCTTTCCGCTCAAA-3') followed by DNA sequencing of size-appropriate bands.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

The number of embryos and samples (n) and definition of statistical significance are indicated in the figure legends. Unpaired t-tests were used to compare means. For multiple comparisons, a One-way ANOVA or Kruskal-Wallis tests were used. For association analyses in gene expression groups, a Fisher’s exact test was used. The criterion for statistical significance was set at $P < 0.05$ and results are represented as mean ± SEM. Mean values were calculated and plotted using GraphPad Prism.