Temporal requirement for SMN in motoneuron development

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Abstract

Proper function of the motor unit is dependent upon the correct development of dendrites and axons. The infant/childhood onset motoneuron disease spinal muscular atrophy (SMA), caused by low levels of the survival motor neuron (SMN) protein, is characterized by muscle denervation and paralysis. Although different SMA models have shown neuromuscular junction defects and/or motor axon defects, a comprehensive analysis of motoneuron development in vivo under conditions of low SMN will give insight into why the motor unit becomes dysfunctional. We have generated genetic mutants in zebrafish expressing low levels of SMN from the earliest stages of development. Analysis of motoneurons in these mutants revealed motor axons were often shorter and had fewer branches. We also found that motoneurons had significantly fewer dendritic branches and those present were shorter. Analysis of motor axon filopodial dynamics in live embryos revealed that mutants had fewer filopodia and their average half-life was shorter. To determine when SMN was needed to rescue motoneuron development, SMN was conditionally induced in smn mutants during embryonic stages. Only when SMN was added back soon after motoneurons were born could later motor axon development be rescued. Importantly, analysis of motor behavior revealed that animals with motor axon defects had significant deficits in motor output. We also show that SMN is required earlier for motoneuron development than for survival. These data support that SMN is needed early in development for motoneuron dendrites and axons to develop normally and that this is essential for proper connectivity and movement.
Introduction

The disease spinal muscular atrophy (SMA) is clinically characterized by proximal muscle weakness and atrophy due to motoneuron dysfunction and eventual loss. Infants with a severe form of the disease exhibit a very low motor unit estimations (MUNE) measurements indicating that the motor unit, that is the motoneuron and all of the muscle fibers it innervates, is compromised. Children with a less severe form of the disease presymptomatically have near normal MUNE but then show decreased MUNE when the disease manifests (1). Interestingly, patients show an upfront manifestation where the most severe symptoms happen in a short amount of time followed by a protracted loss of function over time (2). One explanation for these clinical manifestations is that the motoneurons and perhaps other components of the motor circuit do not develop properly under conditions of low SMN protein leading to their early dysfunction. The best way to test this hypothesis is to analyze motoneurons at the cellular level as they develop under conditions of low SMN.

Analysis in various SMA animal models have revealed defects in the motor unit. Neuromuscular junctions defects and movement defects are seen in mouse, zebrafish, and Drosophila with low SMN (3-9). Transient depletion of SMN in zebrafish and Xenopus leads to motor axon defects (10-12), and presynaptic inputs onto motoneurons are decreased in severe SMA mouse models (13, 14). Analysis of SMA mice has revealed that the spinal motoneurons are born and extend normally into the periphery (15). However, in the most severe SMA mouse model, cranial nerve X does not form correctly and truncations are observed in lumbar spinal nerves (16). In addition, motoneurons and retinal neurons cultured from severe SMA mice have decreased neurite length (17, 18).
Also, in severe SMA mouse models, the neuromuscular junction does not form correctly and there are axonal swellings that are positive for neurofilament (3, 4, 15, 19). Thus, while SMA mouse models do not show such a striking motor axonal defect as in zebrafish, there is an indication that motoneuron development is affected in SMA mice.

It remains unclear, however, how these phenotypes arise and whether defects occur during development or after the motor unit has formed. The best way to assess this is to analyze motoneuron development in live embryos in real time. Due to the accessibility and strength of this phenotype in zebrafish, we can use this system to determine what is needed for motoneurons to develop properly and the role of SMN in this process. Here we combine genetic models, live in vivo imaging and functional analysis to address this issue. To this end, we examined motoneuron development in vivo under conditions of low SMN and used a conditional SMN allele to ask when SMN is needed during motoneuron development for motor function and survival. Our findings show that motoneuron development including motor axon outgrowth, filopodial dynamics as well as dendrite formation are compromised when Smn levels are low. Moreover, these defects cause motor behavior deficits that can be rescued by adding Smn back early in development well before neuritogenesis supporting a need for Smn function during the earliest stages of motoneuron development. We also used the conditional line to ask when SMN is needed in development to fully rescue survival.
Results

Generation of *mz-smn* mutants

To analyze the role of SMN in motoneuron development, we generated a genetic model that depleted SMN from the earliest stages of development. Zygotic *smn* mutants have Smn protein present during the first ~6 days post fertilization (dpf) due to maternal deposition of RNA into the yolk (5). Motoneurons are born around 10 hours post fertilization (hpf) (20), extend their axons out of the spinal cord beginning at ~16-17 hpf (21, 22) and develop dendrites starting around 2 dpf. Therefore, to analyze motoneuron development under conditions of low SMN, we generated mutants lacking both maternal and zygotic SMN by making *maternal:zygotic (mz)-smn* zebrafish mutants. We first needed to rescue zygotic *smn* mutants, which normally die at ~12 dpf so they could grow to adulthood for breeding. Therefore, we generated a transgenic zebrafish line expressing RFP tagged human SMN under the zebrafish *hsp70* promoter (9) Tg(1.5*hsp70I:RFP-Hsa.SMN1)). We generated four unique transgenic lines (line os34-os37) that expressed different levels of RFP-SMN as revealed by heat shocking at 1 dpf and analyzing protein levels at 2 dpf (Fig. 1A). Tg(1.5*hsp70I:RFP-Hsa.SMN1)os36 line was used for further experiments and is hereafter referred to as Tg(hs:RFP-SMN).

To determine if *hsp70* had leaky expression independent of heat shocking, we analyzed RFP-SMN from non-heat shocked embryos and larvae. We detected low levels of RFP-SMN compared to endogenous Smn indicating a low level of RFP-SMN expression in all lines including line os36 independent of heat shock (Fig. 1B). We had previously shown that *smn* +/- zygotic mutants live to ~12 dpf (5). We asked whether this low level of RFP-SMN in non-heat shocked transgenics had any benefit on survival.
Homozygous Tg(hs:RFP-SMN) were crossed to Y262stopΔ29+/- mutant line (hereafter referred to as smn+/-) to generate a transgenic line on the smn mutant background, Tg(hs:RFP- hSMN);smn+/- . We incrossed these fish and performed a survival test. Consistent with previous results, smn-/- mutants lived 11.1 ± 2.2 (mean ± s.d) days; Fig. 1C). However, Tg(hs:RFP-SMN+/-);smn-/- without heat shock lived 17.5 ± 2.0 days (Fig. 1C). This result shows that the presence of leaking RFP-SMN can extend survival and these fish can be used as a more moderate model for SMA.

We next asked whether expressing RFP-SMN in smn mutants could rescue survival. We incrossed Tg(hs:RFP-SMN);smn+/- and selected the transgenic fish by heat shocking them and scoring for RFP at 1 dpf. Starting at 5 dpf these fish were then heat shocked for one hour 3 times a week. We had previously shown that heat shocking every 2-3 days could maintain levels of RFP-SMN (9). After 3 months we genotyped the fish and identified smn+/- fish. Survival analysis revealed that upon repeated heat shock, Tg(hs:RFP-hSMN+/-);smn+/- survived with no early deaths over 30 days. This is in contrast to smn+/- lacking the transgene that lived on average 11 dpf (Fig. 2 A). Although we stopped the survival analysis at 30 dpf, Tg(hs:RFP-hSMN+/-);smn+/- had a normal life span upon consistent heat shock. Analysis of these animals at 12 months revealed indistinguishable body weight and critical swimming speed, a measure of strength/endurance (23), when compared to wild types (Fig. 2 B, C). These results indicate that inducing RFP-SMN can fully rescue smn+/- fish.

Because the RFP-SMN transgene rescued survival upon consistent heat shock, we generated adult smn+/- fish and breed these adults to generate mz-smn mutants. From crosses we obtained mz-smn+/- both with and without the Tg(hs:RFP-SMN) transgene
**mz-smn**⁻/⁻ mutants have abnormal motor axon outgrowth

Previous work in our lab and others has shown that reducing Smn levels in zebrafish using an smn antisense-morpholino (MO) leads to abnormal motor axon outgrowth in morphologically normal embryos (10, 11, 24). Analysis of zygotic smn⁻/⁻ embryos did not reveal motor axon defects because there is a high level of maternal smn in these embryos during the time of motor axon outgrowth (5). Since mz-smn⁻/⁻ embryos lack both maternal and zygotic smn RNA, we asked whether these fish had motor axon defects. We first analyzed mz-smn⁻/⁻ that carried the Tg(hs:RFP-SMN) transgene. Antibody labeling at 28 hpf revealed that ~90% of embryos had motor axon defects especially abnormal branching (Fig. 4 B, D). Motor axon defects were quantitated as previously described (24). To decrease SMN even further, we analyzed mz-smn⁻/⁻ that lacked the transgene.
Scoring motor axons showed that these fish had even more severe motor axon defects with both truncations and abnormal branching present in all embryos (Fig. 4C, D). This was not limited to ventral motor axons as we observed that dorsally projecting motor axons were also abnormal in \textit{mz-smn} mutants both with and without the \textit{Tg(hs:RFP-SMN)} transgene (Supp Fig. 1). This confirms in a genetic model, that motor axon defects are caused by reduction of Smn levels and that the severity of the motor axon defects directly correlates with the levels of SMN.

**Motor axon analysis at the single cell level**

The presence of motor axon defects in \textit{mz-smn} mutants suggests that their motoneurons are not developing normally. To examine this in more detail, we examined individual caudal primary (CaP) motoneurons. For this we injected a DNA construct encoding \textit{mnx1:0.6hsp70:GFP} (9) that randomly labels a few motoneurons per embryo thus allowing detailed analysis of single cells. Analysis of single caudal primary (CaP) motoneurons in wild-type (Fig. 5A-C) and \textit{Tg(hs:RFP-SMN); mz-smn} \textsuperscript{-/-} embryos (Fig. 5 D-F) revealed that motor axonal projections in the mutants lacked robust secondary and tertiary branches although the cell bodies appeared normal. We also noted that SV2 labeling of presynaptic terminals was less robust corresponding to the decreased axonal branching (Fig. 5B and 5E). To quantitate these defects, secondary and tertiary branch length was measured. These data revealed a statistically significant reduction in total branch length (secondary/tertiary combined) in \textit{Tg(hs:RFP-SMN); mz-smn} \textsuperscript{-/-} larvae compared to wild types at 4 dpf (Fig. 6 A-E). We also measured the average branch length and found that it was not significantly different in mutants compared to wild-type
larvae (Fig. 6 F). These data reveal that there are less axonal branches in *mz-smn* mutants, but the branches that are present have a normal length.

Axon branches are generated by actin-rich filopodial that extend off of axons. To examine filopodial dynamics in real-time, we utilized a DNA Lifeact-GFP construct that encodes a peptide from the yeast actin binding protein (Abp) fused to GFP allowing visualization of filopodial actin dynamics without interfering with actin function (25). Moreover, the construct was designed to target the Lifeact to motoneurons (see methods). The Lifeact DNA was injected into 1-2 cell stage *Tg(hs:RFP-SMN); mz-smn*/* or wild-type embryos and filopodial dynamics were analyzed at 28 hpf (Supplementary Movies 1 and 2). Using two-photon microscopy, z-stacks were collected every minute over 60 minutes. Filopodial dynamics were manually measured and the data entered into a computational analysis program (see methods). The analysis revealed that there was a significant decrease in the density of filopodia along CaP motor axons (Fig. 7 A-C). In addition the lifetime of the filopodia was decreased and their rate of retraction was increased (Fig. 7 D, E). These data indicate that actin based filopodial dynamics are severely compromised under conditions of low Smn levels.

Since axons and filopodia were affected, we next wanted to analyze motoneuronal dendrites. These dendrites become evident around 2 dpf and are robust enough to quantify by 4 dpf. Therefore, we analyzed dendrite length in 4 dpf *Tg(hs:RFP-SMN); mz-smn*/* and wild-types. Quantitation of overall dendrite length showed a significant decrease in mutants compared to wild types (Fig. 8 A, B). The average dendrite length was also decreased in mutant larvae (Fig. 8 D) indicating that there are fewer dendrites and those that are present are shorter.
Analysis of Mauthner interneurons, Rohon-Beard sensory neurons and tail sensory neurons did not reveal defects (Supp Fig. 2). However, we did observe a midbrain neuron in the nucleus of the medial longitudinal fascicle (nuc MLF) in the medial lateral (MeL) position that was affected. This neuron sits in the caudal midbrain and projects ipsilaterally into the spinal cord as part of the MLF (26). We found in \textit{mz-smn} mutants that like motoneurons, both the total dendrite length and the average dendrite length for nucMLF midbrain neurons was decreased (Fig. 8 E-H). The proximal nucMLF axonal projection appeared normal; however, we could not observe its distal axonal projection due to labeling by other neurons in the hindbrain and spinal cord. This suggests that other neurons besides motoneurons are affected by low levels of SMN and supports that neurons have different thresholds for SMN (27).

\textbf{SMN is needed early to rescue motor axon defects}

We next set out to address when SMN is needed to rescue motoneuron axon defects. Zebrafish primary motoneurons are born at the end of gastrulation around 10-12 hpf (20, 28) and extend axons out into the periphery around ~16-17 hpf (21, 22). Using conditional induction of SMN, we induced SMN in \textit{Tg(hs:SFP-SMN); mz-smn\textasciitilde} embryos by heat shock at 10, 16, 24 and 27 hpf then assayed motor axon outgrowth. We found that motor axon outgrowth was only rescued when SMN was induced early at the end of gastrulation, 10 hpf (Fig. 9). After this time, motor axons could not be rescued. These data indicate that SMN is needed well before motor axon outgrowth begins for this developmental process to proceed normally.
Only early induction of RFP-SMN in \textit{mz-smn} mutants extends survival

Using this conditional induction of SMN, we also asked when SMN is needed to affect survival. We first determined how long after heat shocking RFP-SMN was detectable. We heat shocked \textit{Tg(hs:RFP-SMN); mz-smn}^-/- embryos at 1 dpf and found that RFP-SMN increased after 30 minutes and started decreasing at \sim 4 days post heat shock. (Supp Fig. 3). This same pattern was observed when we heat shocked at 3 dpf suggesting that RFP-SMN expression after induction was not altered by the age of the fish (Supp Fig. 3).

We then induced once at different times (10-72 hpf) and asked how this affected survival (Fig. 10). We found that heating shocking at 72 hpf was not statistically different than no heat shock (Table 1) and that heat shocking at 36, 48, and 60 hpf while statistically different from no heat shock did not have a dramatic affect on survival. However, heat shocking at 10, 24 or 27 hpf, while not different from one another, did have a significant affect on survival. The difference between adding SMN back at 10 compared to 72 hpf was \sim 2.5 weeks. These data suggest that there is a window in embryonic development where increasing SMN can have affects on survival and that adding SMN back during early embryonic stages has a more dramatic affect than adding back at slightly later embryonic stages.

Since we were seeing a difference between survival when SMN was transiently induced at 24 versus 36 hpf, we next asked whether starting continuous induction of SMN at these times affected survival. For this, we heat shocked every 3rd day starting at either 24 or 36 hpf. We found that if we began continuous SMN induction at 24 hpf, the majority of fish were completely rescued (69% survived for 2 yrs, Fig. 10 B). However, if we waited until 36 hpf to start the continuous SMN induction, survival was only
extended by 4 days (9.6±1.7 no HS (Fig. 10 A) to 13.2±1.4 dpf). These data show that impaired motoneuron development does not lead to decreased survival when SMN is increased in the whole animal.

**Motoneuron defects lead to motor deficits**

SMN is needed early to rescue motoneuron development. However, larvae with rescued motor axons (induction at 10 hpf) and those without rescued motor axons at early time points (induction at 16, 24, 27 hpf) survived for a similar amount of time after transient induction of SMN via heat shock. We wondered whether the motor axon defects affected other SMA phenotypes, in particular motor behaviors that are a critical phenotype in SMA and SMA animal models. To test this, we heat shocked Tg(hs:RFP-SMN); mz-smn−/− embryos at 10 or 24 hpf and then evaluated motor behavior at 5 dpf compared to non-heat shocked Tg(hs-RFP-SMN); mz-smn−/− and heat-shocked wild-type larvae. Consistent with the rescue of motoneuron development (Fig. 9), induction of SMN at 10 hpf fully rescued the gross movement deficit observed in non-heat shocked mz-smn−/− larvae (Fig 11 A). To further determine the nature of the gross motor behavior deficit, we analyzed the frequency with which larvae initiated swimming or turning behaviors and then evaluated the kinematic parameters of each executed swim or turn. Again, we found that induction of SMN at 10 hpf fully rescued the deficit in swim and turn initiations observed in non-heat shocked mz-smn−/− larvae (Fig 11 B). In contrast, induction of SMN at 24 hpf only partially rescued the gross movement and movement initiation deficits observed in mz-smn−/− larvae (Fig 11 A-B), suggesting that the motor axon defects limit functionality of the motor unit. Forward swimming consists of alternating body undulations (each
lateral movement defining a “swim half cycle”) that propel the larva forward. To examine swimming performance, we evaluated the distance moved per swim, the number of swim half cycles, and the average change in body curvature during swimming. Non-heat shocked Tg (hs-RFP-SMN); mz-smn<sup>−/−</sup> showed a significant reduction of all three swimming parameters and induction of SMN in mz-smn<sup>−/−</sup> larvae at either 10 or 24 hpf restored these kinematic deficits equally (Fig 11 C-E). Therefore, early SMN induction that rescues motor axons rescues the initiation and performance of non-evoked, spontaneous swimming and turning behaviors to wild-type levels. This suggests that primary motoneuron fidelity, rescued only by SMN transgene induction at 10 hpf, is more critical for initiating locomotion than for executing swimming once it has been initiated. These data support that motoneuron development defects caused by decreased levels of Smn lead to movement deficits. These data along with the survival data are summarized in Table 2.

**Discussion**

We have used conditional SMN transgenics to ask when SMN is needed for motoneuron development and how this affects movement and survival. We found that removing Smn genetically very early in vertebrate development results in developmental defects in motoneurons. The result is fewer and shorter dendrites and axonal branches leading to decreased axonal and dendritic fields. It is likely that these defects are caused by the observed decrease in filopodial dynamics and filopodial number. The fact that these animals have dramatic movement defects could be due to decreased pre and postsynaptic function because of the dramatically smaller axonal and dendritic fields. This is
supported by the finding that movements are completely restored when axonal defects are rescued. Because we used a conditional allele, we could add SMN back at different times to these animals. Interestingly, the motor axon defects could only be rescued by adding SMN back soon after motoneurons were born and 6-7 hours before motor axon outgrowth. This indicates that SMN is needed as motoneurons develop and not just before they extend their axons thus reflecting a general need for SMN function during the whole motoneuron development process.

Whereas sensory neurons and hindbrain projection neurons did not appear affected in mz-smn mutants, we did observe a midbrain neuron with fewer dendrites and decreased dendritic length. This supports that neurons, and presumably other cells, have certain thresholds for SMN. Decreased proliferation and cell density in the hippocampus has been reported in a severe SMA mouse model supporting that neuronal cell types in addition to motoneurons are affected by low SMN levels (27). Moreover, the dendrite defects in spinal cord motoneurons and nucMLF midbrain neurons were similar suggesting a common mechanism perhaps linked to decreased filopodial dynamics. While sensory axons analyzed here appear morphologically unaffected, we cannot rule out sensory neuron dysfunction. Recent studies have shown that glutamatergic inputs onto motoneurons in certain regions of the spinal cord are decreased in severe mouse models (13, 14). Since the postsynaptic component of these synapses is on dendrites and the cell soma, the decreased number and length of motoneuronal dendrites observed here could account for this decrease in synaptic input. Indeed it has been shown that adding SMN back to motoneurons rescues the glutamatergic inputs onto motoneurons suggesting that this is a motoneuron-autonomous process (29).
Filopodia are actin-based protrusions that drive axonal and dendritic outgrowth. Analysis of filopodial dynamics in live *mz-smn* mutant embryos showed a decrease in the frequency and lifetime of motor axon filopodia. This directly demonstrates in vivo that actin dynamics are disrupted in motoneurons when SMN is decreased. Fewer filopodia lead to defects in axonal branches and thus can explain why there is a decreased axonal arbor in *mz-smn* mutants. Dendrites are also dependent upon filopodia for growth (30). Interestingly, we found that filopodia had shorter half-lives, and thus were less stable, in *mz-smn* mutants. Filopodial stabilization is essential for the formation of dendritic branches and thus could lead to the decrease in dendritic branching seen in *mz-smn* mutants. We had previously shown that the actin binding protein plastin 3 is decreased in SMN mutants (9). It is possible that low plastin 3 levels could affect filopodial actin dynamics. Other studies have also linked SMN and actin pathways (31-33) and could also explain why low levels of SMN causes defects in filopodial actin dynamics.

Using the conditional SMN transgene, we determined when SMN is needed to rescue motoneuron development, movement and survival. Only when SMN was induced at the end of gastrulation and during motoneuron generation were motor axon defects rescued. This indicates that SMN must be present at the onset of motoneuron differentiation for normal motor axon development. If SMN was added back as little as 6 hours later, still at a time before motor axon extension, the motor axon outgrowth defect was not rescued supporting the need for SMN function at the earliest phases of motoneuron development. Thus, we can use motor axon defects as a read-out of aberrant motoneuron development.
A major phenotype of SMA is decreased movement and paralysis. Therefore, we asked whether the developmental defects in motoneurons lead to movement defects. Indeed we found that *mz-smn* larvae had severe movement defects that included decreased frequency of movements (swims and turns), decreased distance moved per swim, decreased swim cycles and decreased body curvature during swim movements. Thus, *mz-smn* larvae initiated fewer movements and when they did swim, they swam less forcefully. Importantly, when SMN was induced by heat shock in *mz-smn* larvae at 10 hpf, motor axons were rescued as was the ability of the fish to swim in all of the above mentioned categories. They were able to initiate swimming and turning movements and swam normally once they moved. When SMN was induced at 24 hpf, the fish still had motor axon defects. In this case overall movement and initiation of movements was somewhat improved, but significantly less than the 10 hpf heat shock group. Body curves and force, however, were fully rescued. These data show that the developmental defects in motoneurons have a significant impact on the ability of the fish to initiate movements. The process of initiating movements is dependent on the nervous system not the muscle and supports that developmental deficiencies in motoneurons, and perhaps other CNS neurons, are responsible for the movement defects.

We also found that we could extend survival of *mz-smn* mutants for ~2.5 weeks with a short pulse of SMN. However, this only occurred if SMN was added before 36 hpf, a time when the nervous system and other organ systems in the animal are developing. This was also supported by our data showing that if we added SMN back continuously starting at 24 hpf, it significantly affected survival while doing so at 36 hpf did not increase survival. Like motoneuron development, there is a window of time
during early development when SMN is needed to impact survival. This is consistent with results from experiments that increase SMN in severe SMA mouse models by a number of approaches (34-38). What we have shown here is that SMN needs to be added back even earlier for normal motoneuron development and movement then for survival. However, even if motor axons and movement are aberrant, adding back SMN early can still increase survival. It is likely that motoneurons continue to require SMN throughout development for normal function. Therefore, adding back SMN at 24 hpf may allow motoneurons to maintain some function even though they have fewer and less robust axonal and dendritic branches. Indeed it has been shown in SMA mice that SMN induction if done early enough had a positive affect on the neuromuscular pathology and the axonal swellings present in these mice suggesting that some abnormalities can be alleviated by addition of SMN (38). Additionally, depleting SMN in motoneurons in severe SMA mice does not dramatically affect survival (39). Consistent with these findings, data presented here demonstrate that optimal motoneuron development, while critical for movement, is not required for survival thereby increasing the optimal therapeutic window for treatment.

Materials and Methods

Generation of maternal zygotic fish

\textit{Tg(hsp70:RFP-SMN^+/_)os36} line (9) was used for generating \textit{mz-smn} mutants.

\textit{Tg(hsp70:RFP-SMN)os36} were crossed to the \textit{smnY262stop^{+/-}} mutant line to generate a transgenic line on the \textit{smn^{-/-}} background \textit{Tg(hsp70:RFP-hSMN^{+/-})os36;smn^{+/-}}.

\textit{Tg(hsp70:RFP-hSMN^{+/-})os36;smn^{+/-}} were then incrossed and heat shocked at 24 hpf in
PCR tubes containing 200 µl of fish water (3 embryos per tube) for 30 minutes at 37°C. Embryos were screened for DsRed fluorescence at 2 dpf. Fish were then heat shocked at 5 dpf in a glass beaker containing 700 ml of fish water for 1 hour at 37°C before transferring to the nursery tank for growing. Heat shocking was repeated 2 times a week. After 3 months, the fish were genotyped. Homozygote mutant fish were selected and outcrossed to wild-type fish to identify heterozygous-Tg(hsp70:RFP-hSMN+/−)os36 or homozygous Tg(hsp70:RFP-hSMN+/+)os36 transgenic fish, which were then kept in, separate tanks. Tg(hsp70:RFP-hSMN+/−)os36;smn−/− or Tg(hsp7:RFP-hSMN+/+)os36;smn−/− were incrossed to generate mz-smn−/− that lacked Smn from both parents and from their own germline.

Survival was determined by incrossing Tg(hsp70:RFP-hSMN+/−);smn+/− followed by heat shocking starting at 1 dpf as described above. Fish carrying the transgene were separated from those lacking the transgene. Fish were allowed to grow and any dead embryos/larvae collected up to 30 dpf. Genotyping was then performed. Although we stopped the survival analysis at 30 dpf, Tg(hsp70:RFP-hSMN+/−);smn+/− live to their normal life span (>2 years in the lab) upon consistent heat shock.

**Timed heat shock induction**

Embryos from crossing Tg(hsp70:RFP-hSMN+/+);os36;smn−/− were collected. At 10, 16, 24, 27, 36 and 48 hpf embryos were placed in PCR tubes contained 200 µl of fish water (3 embryos per tube) and heat shocked at 37°C for 30 minutes. Embryos 24 hpf or older were dechorionated before heat shocking. For continuous SMN induction starting at 24 or 36 hpf, after the first heat shock described above, embryos were then heat shocked at 5
19 dpf in a glass beaker containing 700 ml of fish water for 1 hour at 37°C before transferring to the nursery tank for growing. Heat shocking was repeated 2 times a week and survival analyzed until 30 dpf.

**Western Blotting**

Three zebrafish embryos were placed in 15 μl of blending buffer (62.6 mM Tris pH 6.8, 5 mM EDTA and 10% SDS) and boiled for 10 minutes. The samples were then diluted with an equal volume of loading buffer (100 mM Tris pH 6.8, 4% SDS, 0.2% Bromophenol Blue, 20% glycerol and 200 mM dithiothreitol) and boiled for 2 minutes. A third of each sample from three embryos (~50 μg) were resolved on a 7% polyacrylamide gel. The gel was electrotransfered to Protran BA 83 Nitrocellulose membrane (Whatman). Membranes were probed with SMN mouse monoclonal antibody 2E6 (from Dr. Glenn Morris) or anti-actin (Santa Cruz) (1/5000). Signal was detected with horseradish peroxidase-conjugated goat anti-mouse antibody (1/5000) (Jackson ImmunoResearch Laboratories, Inc.), ECL reagents and Amersham Hyperfilm ECL (Amersham Bioscience).

**Immunofluorescence labeling**

Zebrafish embryos or larvae were anesthetized with tricaine (Sigma, A-5040) then fixed in 4% paraformaldehyde in PBS and 1% DMSO overnight at 4°C. The fish were then washed in 1XPBS for 10 minutes, distilled H$_2$O for 10 minutes followed by a 15 minute incubation at room temperature with -20°C acetone. Samples were then washed with distilled H$_2$O for 20 minutes then incubated overnight at 4°C with znpl (synaptotagmin
2) (40) or anti-SV2 (Developmental Studies Hybridoma Bank, University of Iowa) diluted 1/100 in PBDT buffer and 2.5% normal goat serum. Samples were washed 5 X 10 minutes with PBST at room temperature and incubated overnight at 4°C with Alexa Fluor 488 goat-anti mouse IgG or Alexa Fluor 594 goat-anti mouse IgG (Invitrogen) diluted 1/400 in PBDT and 2.5% normal goat serum. Samples were washed for 5 X 10 minutes in PBST, mounted on a slide with vectashield (Vector Labs, Burlingame, CA, USA) and images captured with a Leica TCS SL scanning confocal microscope system. Motor axons were scored as previously described(24).

**Transient DNA injections for cell and actin labeling**

DNA plasmid *mnx1:0.6hsp70:GFP* (41) was prepared (Qiagen Plasmid Mini kit) and diluted to 50 ng/μl in I-SceI buffer containing 10 mM Tris-HCl, 1 mM dithiothreitol, 10 mM MgCl2, pH 8.8, 5 Units of SceI enzyme (New England Biolab) and 0.1% phenol red. DNA (50 ng/μl) was injected into embryos at the early one-cell stage to 10% of the volume of the cell (~ 1 nl). Injected embryos were transferred into fish water containing penicillin/streptomycin (Invitrogen) 1/100. Injected fish were screened for GFP expressing CaP motor neurons at 26 hpf. Approximately 10% of injected embryos had 1-10 labeled CaP neurons. Best results were obtained when DNA was made fresh monthly.

Lifeact-GFP(25) was generated by fusing the coding sequence for the first 17 amino acids of *Saccharomyces cerevisiae* actin binding protein (Abp)140 to the coding sequence for GFP. The Lifeact-GFP fusion was generated by embedding the nucleotide sequence encoding Lifeact (MGVADLIKKEKSFESKEE) into a PCR primer that was used to amplify GFP. The resulting Lifeact-GFP was subcloned into a 14xUAS-E1b expression
plasmid(42) to generate 14xUAS-E1b:Lifeact-GFP. This was injected along with a motoneuron-specific driver plasmid, tol2-mnx1-3x125bp:Gal4-VP16-tol2 (43) and 100 ng/µl transposase (44) mRNA into embryos at the early one-cell stage to 10% of the volume of the cell (~1 nl). For best results the ratio of the two plasmids was 50 ng/µl (UAS) and 5 ng/µl (GAL4). Injected embryos were transferred into fish water containing penicillin/streptomycin (Invitrogen) 1/100. Injected fish were screened for GFP positive CaP motoneurons at ~28 hpf for two photon imaging. Approximately 5% of injected embryos had 1-5 labeled CaP neurons.

Two-photon time lapse imaging of filopodia

Labeled ~28 hpf embryos were mounted in imaging chambers in 1–2% agarose with 0.05% tricaine and imaged by confocal or two-photon microscopy (45). The embryos were imaged on a custom-built two-photon laser-scanning microscope using a Zeiss 40×/NA 0.8 water-immersion objective. Z-stacks (6 sections spaced at 1 µm) were collected every min over a period of 60 minutes. The Ti:sapphire laser (Chameleon-XR, Coherent, Santa Clara, California) was tuned to 900 nm. At each time-point we collected 20 optical sections spaced at 1.5 µm with a pixel size of 0.167 µm. The highest quality time-lapse sequences were selected for analysis. Fiji was used to measure the lengths of filopodia in each frame of each time-lapse sequence.

Subsequent filopodia analysis was performed using custom written scripts in Matlab (Mathworks). Filopodial lengths were defined as the distance from the base of the filopodium to its tip in maximum intensity projections. The rate of extension/retraction for a given filopodium was defined as the change in length between successive frames.
Filopodial extension/retraction rate for a given motor neuron was calculated as the mean rate of extension or retraction for all filopodia observed on that neuron; these rates were averaged for three axons present in three separate embryos for both wild-type and mutant embryos. The lifetime of an individual filopodium was defined as length of time for which its length was measureable. A filopodium extending from a position at which one had previously disappeared was counted as a second filopodium. The average filopodium lifetime was calculated for each axon and these numbers were averaged for three axons from three embryos for both wild type and mutant embryos. Filopodia density was calculated as the number of filopodia per unit length for each motor neuron averaged over the duration of the time-lapse sequence. These numbers were averaged for three axons from three embryos for both wild type and mutant embryos. Statistical significance was determined in IGOR Pro (Wavemetrics) using the Wilcoxon signed-rank test.

**Length measurements of axon branches and dendrites**

Embryos were fixed in 4% paraformaldehyde in PBS and 1% DMSO overnight at 4°C followed by storage in PBS. Images were captured with the Leica TCS SL scanning confocal microscope system. The motor axon branches at 2 dpf, motor neuron dendrites at 4 dpf or dendrites of the MLF midbrain neurons at 4 dpf were imaged using confocal microscopy (40X objective). All images were set up as 512 x 512 pixels. The microscope calibration information (µm/pixel) was used to convert the Image J measurements (pixel) to microns. The actual size of the images for motor axon branches at 2 dpf was 288.4 µm x 288.4 µm (0.56 µm/pixel), for motoneuron dendrites at 4 dpf was 93.75 µm x 93.75 µm
(0.18 µm/pixel) and for nucMLF dendrites at 4 dpf was 187.5 µm x 187.5 µm (0.36 µm/pixel). All the images were measured using NIH software Image J (Fiji).

Average axon branch or dendrite length was calculated from each neuron and averaged for the scatter plots. Each plotted average was from 100-400 individual dendrites or axon branches.

Zebrafish motor behaviors

To examine spontaneous, non-evoked larval motor behaviors, we captured video recordings using a Motionpro high speed camera (Redlake, Tucson AZ) at 100 or 1000 frames per second with 512 X 512 pixel resolution, using a 50 mm macro lens. Prior to testing for motor behavior, larvae were pre-adapted to the intensity of light in the testing arena for at least 2 hours. Video recordings were initiated 5 minutes after moving larvae to the testing grid in to allow sufficient time for locomotor activity to stabilize.

Behavioral experiments were carried out at 26-27°C and all behavioral measurements were made with the FLOTE software package (46, 47). This software performs tracking of individual larvae and then performs automated analysis of body curvature on each larva to extract kinematic details of swimming and turning movements. The software then classifies each movement according to previously defined kinematic parameters that distinguish discrete motor behaviors. To evaluate gross movement per larva, we simultaneously analyzed the movement of 16 individually housed 5 dpf larvae by recording 80 seconds of continuous video at 100 fps. The total N per experimental group was 32 larvae. To analyze movement initiation and swimming performance, we observed the movement of larvae in a 6 cm dish at a density of 20 larvae per dish. The initiation
and performance of swims and Turns was observed by recording 1 second bouts at 1000 fps and at 4 second intervals for a total of 32 trials. For each larvae, the movement frequency was calculated by dividing the # of turns (or swims) by 32. Swimming performance was evaluated based on the distance moved per swim initiation, and the number of swim half cycles and the average change in body curvature per swim half cycle. A left, then right (or vice versa) body undulation defines 2 swim half cycles.

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The authors declare no conflicts of interest
References


Figure legends

Figure 1: Generation of Tg(hsp70:RFP-SMN) lines.

(A) Western blot showing endogenous Smn and RFP-SMN protein levels from 2 dpf Tg(hsp70:RFP-SMN) os34-37 embryos after heat shocking at 1 dpf. (B) Western blot of endogenous Smn and RFP-SMN without heat shocking in line os36. (C) Survival of smn^-/- (blue line; n=12) and Tg(hsp70:RFP-SMN)os36;smn^-/- (green line; n=7, mean=17.5±2). Data are mean ± s.d.

Figure 2: RFP-SMN fully rescues smn^-/- fish.

(A) Survival of Tg(hsp70:RFP-SMN);smn^-/- heat shocked twice a week (n=21) compared to survival of smn^-/- fish (n=40). (B) Body weight for wild type (WT) and Tg(hsp70:RFP-SMN);smn^-/- (MT). (C) Adult WT and MT were subjected to increasing current (4.1 cm/second steps) every 5 minutes until fatigue as described (Ramesh et al., 2010). Critical swimming speed (U_{crit}, cm/s) was not statistically different between the groups (p>0.5, n=10 for each group). Data are mean ± s.d.

Figure 3: Characterization of mz-smn^-/- fish.

mz-smn^-/- embryos and larvae either with the Tg(hsp70:RFP-SMN) transgene (tg) or without. (A) Lateral view of embryos/larvae at 2, 4, 7, and 10 dpf. (B) Survival of the zygotic smn^-/- fish (red line, n=11, mean =11±2.2), mz-smn^-/- + tg fish (green line, n=9, mean survival=9.6±1.7) and mz-smn^-/- without the transgene (blue line, n=40, mean survival =4). (C) Levels of RFP-SMN independent of heat shock in mz-smn^-/- + tg fish
and (D) \( mz-smn^{+} \) fish. Top blot in D is an overexposure of the RFP-SMN lane. Data are mean ± s.d.

**Figure 4:** Abnormal of motor neurons in \( mz-smn \) mutants.

Lateral views of whole-mount embryos labeled with znp1 antibody at 28 hpf.

Representative images of (A) wild type, (B) \( mz-smn^{+} \) carrying the \( Tg(hsp70:RFP-SMN) \) transgene (tg) and (C) \( mz-smn^{+} \) without the transgene. (D) Motor axon defects were analyzed and embryos characterized as severe, moderate, mild, and no defects.

Significance was determined by Mann–Whitney nonparametric rank test. White arrows denote (A) a normal motor axon (B) a branched motor axon and (C) a truncated motor axon. Scale bar, 50 µm.

**Figure 5:** Single cell analysis of motoneurons.

Wild-type and \( mz-smn^{+} \) containing the \( Tg(hs:RFP-SMN) \) transgene (tg) were injected with \( mnx1:hsp:GFP \) DNA. Confocal images of 4 dpf (A-C) wild-type larvae and (D-F) \( mz-smn^{+} + \) tg larvae. (A, D) GFP labeled CaP motoneurons (B, E) Presynaptic anti-SV2 labeling (C, F) merge. Scale bar, 20 µm.

**Figure 6:** \( mz-smn \) mutants have fewer motor axon branches.

Wild-type and \( mz-smn^{+} \) containing the \( Tg(hs:RFP-SMN) \) transgene (tg) were injected with \( mnx1:hsp:GFP \) DNA. Confocal images were obtained of 2 dpf and motor axons and branches were traced using NIH image J software Fiji. (A) wild-type GFP labeled CaP motoneuron (B) Traced branches from A. (C) \( mz-smn^{+} + \) tg GFP labeled CaP
motoneuron (D) Traced branches from C. (E) Total branch length (mean ± SD) of wild type (WT, n= 7 neurons) and \( mz-smn^{−/−} + \) tg (mutant, n=9 neurons). ***p < 0.0001, two-tailed Students t-test, ns= not significant. Scale bar, 25 µm.

**Figure 7:** \( mz-smn \) mutants have fewer and less stable motor axon filopodia.

1-cell stage wild type and \( mz-smn^{−/−} \) embryos containing the Tg(hs:RFP-SMN) transgene (tg) were injected with Lifeact-GFP DNA targeted to motoneurons (see methods). At ~ 28 hpf motor axons (n=3 motor axons from three different wild-type or mutant embryos) were imaged using 2-photon microscopy and time-lapse sequences used for analysis of filopodial dynamics. (A) wild-type Lifeact-GFP labeled CaP motoneuron (B) \( mz-smn^{−/−} + \) tg Lifeact-GFP labeled CaP motoneuron. (C) Filopodia number per 10 µm of axon (D) Filopodial lifetime (E) Filopodial rates of extension and retraction. *p< 0.01, Wilcoxon signed-rank test. Scale bar, 10 µm.

**Figure 8:** Abnormal motoneuron and nucMLF dendrites.

Confocal images of 4 dpf (A) wild-type, (B) \( mz-smn^{−/−} + \) tg injected with mnx1:hsp:GFP DNA showing GFP labeled CaP motoneurons and a nucMLF midbrain neuron in Tg(mnx1:hsp:GFP) (E) wild-type and (F) \( mz-smn + \) tg larvae. Dendrites were traced using Fiji software. (C) Total and (D) average dendritic length of motoneurons. (G) Total and (H) average dendritic length of nucMLF midbrain neurons. n= 12 neurons analyzed for each category (mean ± SD). Average dendrite length (mean ± SEM) was plotted for each neuron. *** p<0.0001, **p<0.005 two-tailed Student’s t-test. Scale bar, 8 µm for A, B and 15 µm for E, F (main images). Arrows in inset denote axons, arrowhead denote
dendrites.

**Figure 9:** Motor axon defects in *mz-smn* mutants are rescued by early SMN induction. Representative images of znpl labeled ~28 hpf (A) wild-type (B) *mz-smn*+/− + tg without heat shock (hs) (C) *mz-smn*+/− + tg heat shocked at 10 hpf (D) *mz-smn*+/− + tg heat shocked at 16 hpf (E) *mz-smn*+/− + tg heat shocked at 24 hpf (F) *mz-smn*+/− + tg heat shocked at 27 hpf. (G) Motor axon defects were analyzed and embryos characterized as severe, moderate, mild, and no defects. Significance was determined by a Mann–Whitney non-parametric rank test. ns, not significant. Scale bar, 25 µm.

**Figure 10:** Early induction of SMN extends survival. (A) Kaplan-Meier survival plots of *mz-smn*+/− + tg embryos/larvae after heat shock at different time points. No heat shock (dark purple, n=10, mean = 9.6±1.7), heat shock at 36 hpf (dark blue, n=15, mean=13.8±1.6), heat shock at 48 hpf (green, n=11, mean=12.4±1.7), heat shock at 60 hpf (black, n=14, mean=14±1.3), heat shock at 72 hpf (yellow, n=15, mean=10±1.8). Heat shock at 10 hpf (red, n=8, mean=27.1±1.7), heat shock at 24 hpf (light blue, n=8, mean=26.2±1.7), heat shock at 27 hpf (pink, n=10, mean=24.7±2.6). See Table 1 for statistics. (B) Kaplan-Meier survival plots of *mz-smn*+/− + tg embryos after continuous induction starting at 24 hpf (light blue, n=29, 69% survival > 2yrs) or 36 hpf (dark blue, n=24, mean 13.2 ± 1.4).

**Figure 11:** Motor axon defects lead to motor deficits
(A) Mean gross movement of larvae over a period of 80 seconds. (B) Mean frequency (percentage of recorded trials) in which larvae initiated swimming and turning behaviors. 

(A-B) N = 32 larvae per group. Evaluation of swimming kinematic performance was determined by measuring (C) distance moved per swim, (D) number of swim half cycles, and (E) mean body curvature change per swim half cycle. For this ~30 fish/group were recorded and the following number of swims analyzed: WT No HS, 79; WT HS 10 hpf, 85; WT HS 24 hpf, 90; Tg (hs-RFP-SMN); mz-smnΔ/Δ No HS, 23; Tg (hs-RFP-SMN); mz-smnΔ/Δ HS 10 hpf, 104; Tg (hs-RFP-SMN); mz-smnΔ/Δ HS 24 hpf, 40. *p<0.05, **p<0.01, ***p<0.001, one-way ANOVA. Error bars denote SEM. HS= heat shock.
**Tables**

**Table 1:** SMN has a positive affect on survival when added back early in development.

Statistics for survival analysis after SMN-RFP induction at different developmental times. P values were calculated from Kaplan-Meier data and log rank-test using Chi-Square analysis. *** p<0.001; ns, not significant.

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Table 2: SMN is required earlier for motoneuron development than for survival. SMN was induced either once or continuously and assays were performed as described in the text. Motor axons were analyzed at 28 hpf and motor behavior analysis performed at 5 dpf. nd, not determined.

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Abbreviations

mz, maternal zygotic

hpf, hours post fertilization

dpf, days post fertilization

SMN, survival motor neuron (Smn, for zebrafish)

SMA, spinal muscular atrophy