Temporal requirement for SMN in motoneuron development

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Received December 17, 2012; Revised and Accepted February 27, 2013

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 of 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 number estimation (MUNE) measurements indicating that motor units, that is the motoneuron and all of the muscle fibers it innervates, are compromised. Children with a less severe form of the disease pre-symptomatically 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 survival motor neuron (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 of various SMA animal models has revealed problems with the motor unit. Neuromuscular junction defects and movement deficits are seen in the 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

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SMA mice has revealed that 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, 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 determine when SMN is needed during motoneuron development for proper 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 neurite outgrowth, filopodial dynamics as well as dendrite formation are rescued 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 h post fertilization (hpf) (20), extend their axons out of the spinal cord beginning at ~16–17 hpf (21,22) and develop dendrites starting ~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(1.5hsp70I:RFP-Hsa.SMN1). We generated four unique transgenic lines (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(1.5hsp70I: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 with 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 with smn1b229+/- 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 ± SD) 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 1 h three 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. 2A). 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 with wild-types (Fig. 2B and 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 (Fig. 3A). $mz-smn^{-/-}$ embryos/larvae with the transgene had no zebrafish Smn but did have a low level of RFP-SMN without heat shocking due to the low level of leaky transgene expression (Fig. 3C) and lived 9.2 ± 0.6 dpf, $n = 10$ (Fig. 3C, green line). $mz-smn^{-/-}$ embryos/larvae without the transgene had no detectable zebrafish Smn nor any detectable RFP-SMN (Fig. 3D). These fish looked indistinguishable from wild-types at 1 dpf, but all died at 4 ± 0 dpf (Fig. 3B, blue line). These data support that the Smn in zygotic $smn^{-/-}$ is maternally derived and can be removed by generating $mz-smn$ mutants. Moreover, we show that low levels of RFP-SMN can extend survival of $mz-smn$ mutants.

$mz-smn^{-/-}$ 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 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. 4B and 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 and D). This was not limited to ventral motor axons as we observed that dorsally projecting motor axons were also abnormal in $mz-smn$ mutants both with and without the $Tg(hs:RFP-SMN)$ transgene (Supplementary Material, Fig. S1). This confirms in a genetic model that motor axon defects are caused by the reduction in Smn levels and that the severity of the motor axon defects directly correlates with the levels of SMN.

![Figure 2. RFP-SMN fully rescues $smn^{-/-}$ fish. (A) Survival of $Tg(hs70:RFP-SMN);smn^{-/-}$ heat shocked twice a week ($n = 21$) compared with survival of $smn^{-/-}$ fish ($n = 40$). (B) Body weight for wild-type (WT) and $Tg(hs70:RFP-SMN);smn^{-/-}$ (MT). (C) Adult WT and MT were subjected to increasing current (4.1 cm/s steps) every 5 min until fatigue as described (23). 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 ± SD.](http://hmg.oxfordjournals.org/)

Motor axon analysis at the single cell level

The presence of motor axon defects in $mz-smn$ mutants suggests that their motoneurons were not developing normally. To examine this in more detail, we examined individual caudal primary (CaP) motoneurons. For this, we injected a DNA construct encoding $mxn1:0.6hs70:GFP$ (9) that randomly labels a few motoneurons per embryo thus allowing detailed analysis of single cells. Analysis of single CaP motoneurons in wild-type (Fig. 5A–C) and $Tg(hs:RFP-SMN);mz-smn^{-/-}$ embryos (Fig. 5D–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...
dense corresponding to the decreased axonal branching (Fig. 5B and E). To quantitate these defects, secondary and tertiary branch lengths were measured. These data revealed a statistically significant reduction in total branch length (secondary/tertiary combined) in Tg(hs:RFP-SMN); mz-smn\(^2/2\) larvae compared with wild-types at 4 dpf (Fig. 6A–E). We also measured the average branch length and found that it was not significantly different in mutants compared with wild-type larvae (Fig. 6F). 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 filopodia 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 inferring with actin function (25). Moreover, the construct was designed to target the Lifeact to motoneurons (see Materials and Methods). The Lifeact DNA was injected into 1–2 cell stage Tg(hs:RFP-SMN); mz-smn\(^2/2\) or wild-type larvae (Fig. 6A–E). These data reveal that there are less axonal branches in mz-smn mutants, but the branches that are present have a normal length.

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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 at \( \sim 10-12 \) hpf (20,28) and extend axons out into the periphery around \( \sim 16-17 \) hpf (21,22). Using conditional induction of SMN, we induced SMN in \( Tg(hs:RFP-SMN); mz-smn^{-/-} \) embryos by heat shock at 10, 16, 24 and 27 hpf and 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 \( mz-smn \) mutants extends survival

Using this conditional induction of SMN, we next asked when is SMN needed to affect survival. We first determined how long after heat shocking RFP-SMN was detectable. We heat shocked \( Tg(hs:RFP-SMN); mz-smn^{-/-} \) embryos at 1 dpf and found that RFP-SMN increased after 30 min and started decreasing at \( \sim 4 \) days post heat shock (Supplementary Material, Fig. S3). 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 (Supplementary Material, Fig. S3). 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 non-heat shocked animals (Table 1) and that heat shocking at 36, 48 and 60 hpf while statistically different from non-heat shocked did not have a dramatic affect on survival. However, heating 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 with 72 hpf was \( \sim 2.5 \) weeks. These data suggest that there is a window in embryonic development where increasing SMN can have effects on survival and that adding SMN back during early embryonic stages has a more dramatic effect 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 third 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 years, Fig. 10B). However, if we waited until 36 hpf to start the continuous SMN induction, survival was only extended by 4 days \([9.6 \pm 1.7 \text{ no heat shock (Fig. 10A) to 13.2 } \pm 1.4 \text{ dpf}]\). These data show that impaired motoneuron development, as seen at 24 and 27 hpf, 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 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. These data show that impaired motoneuron development, as seen at 24 and 27 hpf, does not lead to decreased survival when SMN is increased in the whole animal.
swimming or turning behaviors and then evaluated the kine-
matic 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. 11B). In contrast, induction
of SMN at 24 hpf only partially rescued the gross movement
and movement initiation deficits observed in mz-smn\(^{-/-}\)
larvae (Fig. 11A–B), suggesting that the motor axon defects
limit functionality of the motor unit. Forward swimming con-
sists of alternating body undulations (each lateral movement
defining a ‘swim half cycle’) that propel the larva forward.
To examine swimming performance, we evaluated the dis-
tance 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\(^{-/-}\) larvae at either 10 or 24 hpf
restored these kinematic deficits equally (Fig. 11C–E). There-
fore, 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 sug-
gests 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 summar-
ized in Table 2.

DISCUSSION

We have used conditional SMN transgenes to ask when SMN
is needed for motoneuron development and how this affects
movement and survival. We found that removing Smn genet-
ically very early in vertebrate development results in develop-
mental 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 func-
tion because of the dramatically smaller axonal and dendritic
fields. This is supported by the finding that swimming and
turning 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,
6–7 h 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 some 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 prolif-
eration 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 the sensory axons analyzed here
appear morphologically unaffected, we cannot rule out sensory

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 \(\mu\)m.
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 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).

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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 swim 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 were 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 the development of 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 effect 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**

The Tg(hsp70:RFP-SMN+/−)os36 line (9) was used for generating mz-smn mutants. Tg(hsp70:RFP-SMN)os36 were crossed with the smn1fh229/+ . Tg(hsp70:RFP-hSMN+/−)os36;smn+/− were then incrossed and heat shocked at 24 hpf in PCR tubes containing 200 μl of fish water (three embryos per tube) for 30 min 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 h at 37°C before transferring to the nursery tank for growing.
Heat shocking was repeated two times a week. After 3 months, the fish were genotyped. Homozygote mutant fish were selected and outcrossed with 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(hsp70: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 (three embryos per tube) and heat shocked at 37°C for 30 min. 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 dpf in a glass beaker containing 700 ml of fish water for 1 h at 37°C before transferring to the nursery tank for growing. Heat shocking was repeated two 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 min. 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 min. A third of each sample from three embryos (~50 µg) were resolved on a 7% polyacrylamide gel. The gel was electrotransferred 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) and then fixed in 4% paraformaldehyde in PBS and 1% DMSO overnight at 4°C. The fish were then washed in 1× PBS for 10 min, distilled H2O for 10 min followed by a 15 min incubation at room temperature with ~20°C acetone. Samples were then washed with distilled H2O for 20 min and then incubated overnight at 4°C with znpl1 (synaptotagmin 2) (40) or anti- SY2 (Developmental Studies Hybridoma Bank, University of Iowa) diluted 1/100 in PBDT buffer and 2.5% normal goat serum. Samples were then washed 5 × 10 min 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 Ig (Invitrogen).

**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 the 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). ***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.
diluted 1/400 in PBDD and 2.5% normal goat serum. Samples were washed for 5 × 10 min 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 Scel 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 (MGVADLIKKFE-SISKEE) 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-mnx1-3 × 125-bp: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

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**Figure 9.** Motor axon defects in *mz-smn* mutants are rescued by early SMN induction. Representative images of znpl1-labeled ~28 hpf (A) wild-type, (B) *mz-smn*+/− + tg heat shocked at 10 hpf, (C) *mz-smn*+/− + tg heat shocked at 16 hpf, (D) *mz-smn*+/− + tg heat shocked at 24 hpf, and (E) *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.
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% tricine 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 (six sections spaced at 1 μm) were collected every min over a period of 60 min. The Ti:sapphire laser (Chameleon-XR, Coherent, Santa Clara, CA, USA) 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 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 motoneuron 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. Statistic 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, motoneuron dendrites at 4 dpf or dendrites of the nucMLF midbrain neurons at 4 dpf were imaged using confocal microscopy (×40 objective). All images were set up as 512 × 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 × 288.4 μm (0.56 μm/pixel), for motoneuron dendrites at 4 dpf was 93.75 × 93.75 μm (0.18 μm/pixel) and for nucMLF dendrites at 4 dpf was 187.5 × 187.5 μm (0.36 μm/pixel). All the images were measured using NIH software Image J (Fiji).

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<th>Table 1. SMN has a positive affect on survival when added back early in development</th>
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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 the Chi-square analysis. ***P < 0.001; ns, not significant.
Average axon branch or dendrite length was calculated from each neuron and averaged for the scatter plots. Each plotted average was from 100 to 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...
Table 2. SMN is required earlier for motoneuron development than for survival

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<td>36 hpf</td>
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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.

camera (Redlake, Tucson, AZ, USA) at 100 or 1000 frames per second with 512 × 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 h. Video recordings were initiated 5 min 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 the 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 s 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 s bouts at 1000 fps and at 4 s intervals for a total of 32 trials. For each larva, 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 two swim half cycles.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS

We thank the fish facility staff for excellent fish care and Dr Alison Lyon for reading the manuscript. The mnx1-3 × 125-bp:Gal4-VP16 plasmid was a kind gift from Dr Juan Bruses (University of Kansas Medical Center) and the 2E6 SMN monoclonal antibody was a kind gift from Dr Glenn Morris. We would also like to thank Ms. Laura Brennan, research scientist at Upper Arlington High School, for her commitment to science education and her excellent mentorship of young scientists.

Conflict of Interest statement. None declared.

FUNDING

This work was funded by NIH (RO1 NS050414) to C.E.B., NSF (IOS-0920357) to J.D.J. and NIH grant (RO1 MH092257) to M.G. Additional support was provided by NIH P30 NS045758.

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