Neuron-specific knock-down of SMN1 causes neuron degeneration and death through an apoptotic mechanism

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Abstract

Spinal muscular atrophy is a devastating disease that is characterized by degeneration and death of a specific subclass of motor neurons in the anterior horn of the spinal cord. Although the gene responsible, survival motor neuron 1 (SMN1), was identified 20 years ago, it has proven difficult to investigate its effects in vivo. Consequently, a number of key questions regarding the molecular and cellular functions of this molecule have remained unanswered. We developed a Caenorhabditis elegans model of smn-1 loss-of-function using a neuron-specific RNA interference strategy to knock-down smn-1 selectively in a subclass of motor neurons. The transgenic animals presented a cell-autonomous, age-dependent degeneration of motor neurons detected as locomotory defects and the disappearance of presynaptic and cytoplasmic fluorescent markers in targeted neurons. This degeneration led to neuronal death as revealed by positive reactivity to genetic and chemical cell-death markers. We show that genes of the classical apoptosis pathway are involved in the smn-1-mediated neuronal death, and that this phenotype can be rescued by the expression of human SMN1, indicating a functional conservation between the two orthologs. Finally, we determined that Plastin3/plst-1 genetically interacts with smn-1 to prevent degeneration, and that treatment with valproic acid is able to rescue the degenerative phenotype. These results provide novel insights into the cellular and molecular mechanisms that lead to the loss of motor neurons when SMN1 function is reduced.

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Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive neurodegenerative disease and one of the most common genetic causes of infant mortality (1). The disease, for which no effective treatment is currently available (2), is characterized by the specific loss of lower spinal cord motor neurons, leading to the atrophy of innervated muscles, paralysis and ultimately death (3). Based on age of onset and motor disabilities, SMA can be clinically subdivided into three types of varying severity (4), all of which are associated with deletions or point mutations in the survival motor neuron 1 gene [SMN1 (5)], which has been mapped to a highly unstable, telomeric region of chromosome 5 (Sq19) (6). In addition to SMN1, humans have a nearly identical gene called SMN2; however, the presence of a C-to-T mutation within exon 7 of SMN2 gives rise to a truncated protein, which is rapidly degraded (7). As SMN2 is present in different copy numbers in the genome and is able to produce very low levels of functional protein (5–10% of the total SMN), it acts solely as a genetic modifier when SMN1 is altered, and the level of its expression is responsible for the varying severity of the disease (8).

SMN proteins are ubiquitously expressed both within and outside the nervous system (9). SMN is a component of the well-characterized Gemin complex, which is involved in a series of basic cellular processes, including the biogenesis and assembly of small nuclear ribonucleic proteins and pre-mRNA splicing and transcription (10,11). SMN also plays a role in axonal growth and neuromuscular junction formation, as well as axonal mRNA transport, sub-cellular localization and/or local translation (12,13). However, the specific loss of spinal cord motor neurons in SMA patients cannot be explained simply in terms of their SMN expression pattern, and it is not completely clear which of the SMN functions is responsible for the specific degeneration and death of motor neurons (14).

Modeling SMA in animal systems is therefore crucial to further understand the pathogenesis of the disease and the function played by SMN in motor neurons, as well as to identify potential therapies. Several vertebrate and invertebrate models have been developed, which have provided important information on the changes in the nervous system that occur in response to loss of SMN1 (13,15–17). However, the absence of a second SMN gene in the genome of all animal models, including Caenorhabditis elegans, makes it very difficult to investigate the in vivo role of SMN1 due to the early lethality of SMN1 knockouts (13,15,16,18–20), hindering the ability to visualize and study this form of neuronal degeneration. In particular, in the current animal models of SMA it has not been possible to determine in vivo the steps that lead to neuron degeneration (13) and, with few exceptions (19,21), it has not been possible to observe neuronal death when SMN1 function is reduced or absent.

Caenorhabditis elegans has a single gene homologous to human SMN, smn-1, which is ubiquitously expressed during all developmental stages (18). The nematode SMN protein exhibits an overall sequence homology and a conserved domain topology with its human counterpart (18). Moreover, the basic functions of SMN, including the ability to self-associate and bind to partner proteins, are conserved in C. elegans (18,22). As observed in other species, a genetic deletion of smn-1 (null allele ok355) causes strong larval lethality (20). The very few surviving ok355 homozygous larvae (escapers) show impaired locomotion and pharyngeal pumping defects, but not neuronal degeneration (20,23). Similar phenotypes have been observed after systemic RNA interference [RNAi (18,22)]. Finally, a hypomorphic mutant allele (cb131) with a single amino acid substitution (D27N) that mimics a point mutation found in some SMA patients (D44V) has been generated (24). Although this mutation overcomes the viability problems of the null mutant, the muscle and nervous system architecture are not affected and there is little change in motility.

Here, we report a novel SMA model in C. elegans. Using cell-specific RNAi knock-down (25), we successfully generated viable transgenic strains in which the function of smn-1 is specifically reduced in a subclass of motor neurons. These transgenic animals mimic some key features of SMA, including impaired locomotion, neuronal degeneration and neuronal death. We report, for the first time, the early steps in neuron degeneration and the loss of neurons due to smn-1 depletion in vivo. We reveal that the neuronal degeneration and death induced by smn-1 reduction are cell-autonomous and proceed in part through an apoptotic mechanism. Furthermore, we investigate the conserved neural protective function of SMN1 from nematodes to humans, and reveal that Plastin3/plst-1 is a genetic interactor of SMN1 in neuronal death. Finally, we show that valproic acid (VPA) is able to rescue the degeneration of C. elegans smn-1-depleted neurons.

Results

Cell-specific knock-down of smn-1 in D-type motor neurons causes movement defects and loss of targeted neurons

A transgene-driven RNAi strategy (25) was used in C. elegans to knock-down the essential gene smn-1 selectively in the 19 D-type gamma-aminobutyric acid (GABA)ergic motor neurons (punc-25:smn-1 MNs RNAi) (26). The smn-1 silenced strains obtained were viable, fertile, and did not present any visible developmental defects. Given the role of D-type motor neurons in the regulation of C. elegans backward movement (26), we tested this locomotion behavior. As a negative control, we used animals in which we had silenced, under the same conditions and in the same cells, kal-1, a C. elegans neurodevelopmental gene that is not normally expressed in the D-type motor neurons (27). We found that the normal backward response was highly reduced in smn-1 silenced animals (27%), compared with non-silenced controls (95%) and kal-1 silenced animals (89%) (P < 0.001; Fig. 1A and Supplementary Material, Table S1). In contrast, the forward movement was unaffected in all three strains (data not shown), suggesting that the muscles and other classes of motor neurons were not compromised. To confirm that the defect in backward movement was the result of D-type motor neurons dysfunction, we analyzed the thrashing behavior of the smn-1 knocked-down animals that requires functional cholinergic neurons (28). We found that thrashing was not altered in the smn-1 knocked-down animals (Supplementary Material, Fig. S2), indicating that the RNAi had not spread to other tissues.

We next investigated the effect of smn-1 knock-down on the survival of the 19 D-type motor neurons. We transferred the smn-1 knock-down transgene to a strain that expressed GFP in these cells (oxyIs12 [punc-47::GFP]) (Fig. 1D). smn-1 knock-down induced a dramatic reduction in the number of visible GFP-labeled D-type motor neurons, with an average of 1.8 neurons observed per young adult animal (Fig. 1B and F and Supplementary Material, Table S3); no defects were observed in non-silenced controls or in kal-1 silenced animals (average of 19 and 18.7 neurons/animal, respectively; Fig. 1B and D and Supplementary Material, Table S3). A very low but significant reduction of visible motor neurons was also observed after...
classical systemic RNAi of smn-1 (Supplementary Material, Table S3). We confirmed that the knock-down did not spread to nearby cholinergic motor neurons in the ventral cord by using a strain in which the D-type motor neurons expressed RFP and the cholinergic neurons expressed GFP (vsIs48[punc-17::GFP]; punc-47::RFP). smn-1 knock-down induced a strong reduction of visible D-type motor neurons but no alteration in the number of visible cholinergic neurons (Supplementary Material, Fig. S4 A–C).

To further characterize this apparent neuronal loss, we investigated the axonal morphology and synaptic distribution of D-type motor neurons, as their alteration could be early signs of a degenerative process. D-type motor neurons are located in the ventral cord and each neuron extends a circumferential commissure to the dorsal side of the animal (26); these axonal processes can be visualized in the transgenic strain described above (oxIs12) (Fig. 2A). In the few surviving GFP-labeled motor neurons of smn-1 silenced animals, we observed a low but significant defect in axon morphology consisting of extra branching and guidance defects (7% defective commissures; P < 0.001, non-parametric Mann–Whitney test) (Fig. 2B and C and Supplementary Material, Table S5). Presynaptic densities can be visualized using the vesicle-associated Ras GTPase, RAB-3, fused to the mCherry fluorescent protein (vdIs4[punc-25::ChFP::RAB-3]) (29). We combined the smn-1(MNs RNAi) with the transgenes to visualize the D-type motor neurons with GFP (oxIs12) and their presynaptic loci with mCherry (vdIs4). In non-silenced animals, RAB-3 was continuously visible in the ventral and the dorsal cord in all animals, with a punctate pattern (Fig. 2E). On the contrary, in 92% of smn-1 knocked-down animals we observed a defect in RAB-3 localization (Fig. 2F and Supplementary Material, Fig. S6). Defective localization of RAB-3 consisted of gaps in the punctate pattern, of different length/severity, and sometimes the complete absence of RAB-3 protein in the entire cord (Supplementary Material, Table S5).
Figure 2. *smn-1* RNAi knock-down causes defects in D-type motor neurons axons and synapses. (A–G) RNAi knock-down of *smn-1* in D-type motor neurons produces changes in motor neuron axonal morphology. In *oxIs12 (pMNs::GFP)* transgenic animals motor neuron axons are visible. Anterior is right and ventral is down. In control animals (A) commissures are directed to the dorsal site (up) as single axons (arrowheads). *smn-1* knock-down (B, C) induces axonal branching and defects in axonal guidance. Transgenic strains were obtained using a medium concentration of interfering construct. (D–G) *vdIs4 (pMNs::ChFP::RAB-3), oxIs12 (pMNs::GFP)* double transgenic animals allow visualization of the localization of RAB-3, a presynaptic marker, in the ventral cord (E,G) and the motor neuron cytoplasm (D,F), respectively. White asterisks indicate motor neuron cell bodies, where RAB-3 is not localized. Anterior is left and ventral is down. In the upper panels the ventral cord is observed with epifluorescence using a GFP filter, and in the lower panel it is observed using a Texas Red filter. (E) Localization of RAB-3 in motor neurons in control animals. (G) Localization of RAB-3 in animals knocked-down for *smn-1*. A small gap of presynaptic RAB-3 (white arrowhead) is visible in the ventral cord, where axons are still present in the corresponding area in the upper panel. (H) Average number of motor neurons visible after *smn-1 (MNs RNAi)* knock-down and in controls at L1, the first larval stage after hatching. At this stage, six D-type neurons express GFP in the wild-type animals. Each bar represents the mean number of visible cells ± S.E.M.; >30 animals were observed. * indicates significantly different from control (*P* < 0.005, non-parametric Mann–Whitney test). (I) Percentage of visible motor neurons that correctly express RAB-3 at L1 stage. >30 animals were observed. * indicates significantly different from control (*P* < 0.001, non-parametric z statistic test). Transgenic strains in (F–I) were obtained using a high concentration of interfering construct.
Material, Fig. S6), which suggests a progressive loss of RAB-3 protein at the presynaptic site. To establish whether this defect precedes neuron disappearance, we focused on D-type motor neurons expressing GFP and analyzed RAB-3 localization only in these cells. As very few D-type motor neurons are visible in smn-1 knock-downed animals at the adult stage (1.8 neurons per young adult animal; Fig. 1B and Supplementary Material, Table S3), we analyzed younger animals (L1 stage) that presented an average of 3.5 visible neurons per animal (Fig. 2H). All the GFP-labeled cells expressed RAB-3 in L1 non-silenced animals (Fig. 2I). In contrast, in smn-1 knock-downed animals only 45% of the remaining GFP-labeled neurons still correctly expressed the RAB-3 marker. These results reveal that the earliest observable defect of smn-1 knock-down is a reduced number of presynaptic densities, followed by morphological alterations in the axon and loss of cytoplasmic expression of GFP.

**smn-1 knock-down causes neuronal death through an apoptotic mechanism**

To determine whether loss of GFP corresponded to cell death, we first investigated whether apoptosis could be detected using Differential Interference Contrast (DIC) microscopy. In C. elegans, programmed cell death causes a characteristic change in cell morphology and refractility that can be observed in living animals using DIC microscopy (30,31). This unique feature made it possible to genetically dissect for the first time the programmed cell death pathway in C. elegans (30). We found that in smn-1(MNs RNA) animals, some neurons lacking GFP expression presented an altered morphology of the nucleus, with a button-like shape characteristic of apoptotic corpses (Fig. 3A and C and Supplementary Material, Table S7A). Occasionally, the apoptotic appearance was also observed in neurons that were still faintly GFP fluorescent (Fig. 3B and D). A further marker of cell death is the presence on the surface of dying cells of ‘eat-me’ signals recognized by engulfing cells. One such signal is the tranglrytalin-like secreted protein TTR-52 (32). We took advantage of a transgenic strain expressing TTR-52 fused to the red fluorophore mCherry (smIs119[php-16.2:::TTR-52::ChFP]), which specifically labels apoptotic dying cells before engulfment (32). In smn-1(MNs RNA); smIs119 double transgenic animals, we observed TTR-52-positive signals with the typical ring shape in the nerve cord (Fig. 3F and H and Supplementary Material, Table S7B). The fragmentation of chromosomal DNA is a late event of apoptosis and can be visualized using the TUNEL assay. This assay revealed few positive spots in the ventral nerve cord when smn-1 was knocked-down (Fig. 3I and L).

These findings, which were never observed in control animals (Fig. 3I and K and Supplementary Material, Table S7B), indicate that smn-1 knock-down causes the degeneration and death of D-type motor neurons, at least in part through an apoptotic mechanism.

**smn-1-silenced dying D-type motor neurons acquire fluorescence**

While analyzing smn-1 silenced animals carrying the smIs119[php-16.2:::TTR-52::ChFP] transgene, we unexpectedly observed a faint but clear and reproducible accumulation of green fluorescence in D-type motor neurons (Fig. 3M and P). These animals did not carry any reporter expressing GFP in neurons. In some cases, the red fluorescent ‘eat-me’ signal of the TTR-S2::ChFP transgene surrounded a cell that was accumulating green fluorescence (Fig. 3M–R). Moreover, some of these cells presented the refractile, button-like structure typical of apoptotic dying cells (Fig. 3S–X). The smn-1 knock-down transgenic strain carried as the selection marker a GFP reporter (GB301 pcs-2::gfp), which is specifically expressed in 17 cell nuclei in the pharynx (33). This reporter is never expressed in D-type motor neurons. Analysis of the emission spectra of the dying fluorescent neurons in the nerve cord with confocal microscopy and immunostaining against GFP unambiguously revealed that the fluorescence observed in these cells was due to GFP (data not shown). A similar acquired green fluorescence was observed when we used a different selection marker expressing GFP in the intestinal nuclei (pM67 pelt-2::gfp) (34) (Supplementary Material, Table S8). Finally, acquired red fluorescence was observed when we used the pcs-2 promoter fused to the red fluorophore DsRed2 (GB300 pcs-2::DsRed2) (Supplementary Material, Table S8). This novel-acquired fluorescence could in principle be due to an uptake by motor neurons of the fluorophore produced by different cells or, more likely, to a gene deregulation in the dying neurons that promotes the leakage expression of the fluorophore from the promoter used as the marker. Although the precise mechanism underlying this phenotype has not yet been elucidated, the accumulation of fluorescent material in D-type motor neurons correlated with their degeneration induced by the knock-down of smn-1. We therefore decided to determine whether this fluorescence could be used as a read-out for smn-1-induced neuronal death, as it would greatly facilitate any further investigation with this strain. To confirm the specificity of this phenotype, we silenced smn-1 in a dose-dependent manner by using different concentrations of interfering transgene (25). At concentrations of 200 ng/ul (high), 10 ng/ul (medium) and 2 ng/ul (low), we observed an average of 5.2, 3.2 and 2.0 green fluorescent neurons per animal in the ventral nerve cord, respectively (high concentration (HC), medium concentration (MC) and low concentration (LC) in Fig. 4A and Supplementary Material, Table S8). Finally, by integrating the knock-down transgene in the genome, the acquired fluorescence was maintained and had the highest penetrance (9.2 green fluorescent neurons per animal), as expected from loss of mosaicism following integration (HC integrated (HCIs) in Fig. 4A and Supplementary Material, Table S8). As an additional negative control, we silenced a gene known to play a function in D-type motor neurons (unc-70), but whose knock-down causes various abnormalities but not cell death (36); in this case, we also never observed fluorescent neurons (Fig. 4A and Supplementary Material, Table S8). As an additional negative control, we silenced a gene known to play a function in D-type motor neurons (unc-70), but whose knock-down causes various abnormalities but not cell death (36); in this case, we also never observed fluorescent neurons (Fig. 4A and Supplementary Material, Table S8). To establish the timing of the acquired fluorescence, we corroborated this phenotype to the presynaptic defect. We focused on D-type dying motor neurons and analyzed RAB-3 localization in these neurons in smn-1 knock-downed animals. Among 460 neurons presenting acquired fluorescence in knocked-down animals (n = 99 animals), 386 (84%) presented a complete absence of RAB-3 protein, strongly suggesting that in almost all cases the fluorescence is acquired after RAB-3 is lost at the presynaptic sites (data not shown).

**Degeneration and death of D-type motor neurons are age-dependent and can be rescued by human SMN1**

SMA is a genetic disease showing a progressive neuron degeneration following an early onset of the disease. Therefore, we investigated whether after smn-1 knock-down the observed
Figure 3. The degeneration of motor neurons after smn-1 knock-down ends with the death of the cell and accumulation of fluorescence. The motor neuron death induced by smn-1(MNs RNAi) can be visualized in four different ways. (A–D) Using DIC microscopy in the ventral cord of smn-1 transgenic animals we observed a button-like structure typical of apoptotic dying cells (white arrowheads) in association with a neuron faintly expressing GFP from the osh12 (pMNs::GFP) transgene. Arrows correspond to viable neuronal nuclei in the ventral cord. (C, D) Enlargements of areas outlined in (A) and (B). Upper images were obtained with visible light, and lower images with GFP filter epifluorescence. (E–H) A TTR-52::ChFP positive neuron, with a typical ring shape, is observed in the ventral cord of smn-1(MNs RNAi), sml119 (TTR-52::ChFP) double transgenic animals (white arrowheads). A quantification of TTR-52::ChFP positive neurons is reported in Supplementary Material, Table S7. (G, H) Enlargements of areas outlined in (E) and (F). Upper panels were obtained with visible light, and lower panels were obtained with Texas Red filter epifluorescence. (I–L) Apoptotic dying neurons in the ventral cord were analyzed in permeabilized animals using TUNEL assay. smn-1(MNs RNAi) knocked-down animals, but not controls, presented TUNEL-reactive cells along the ventral cord. (I) Control animal, adult stage, presenting no labeling in the cord. (J) smn-1(MNs RNAi) animal, adult stage, presenting a positively labeled cell in the cord. (K) Enlargement of the area outlined in J, showing the labeled cell in the cord (arrow). n = 40 for control and for smn-1(MNs RNAi). White asterisks correspond to the vulva, which is always labeled. All images were taken with visible light and DIC. (M–R) Accumulation of fluorescence is sometimes surrounded by a TTR-52::ChFP ring (white arrowheads), in smn-1(MNs RNAi), sml119 (TTR-52::ChFP) double
pheno-
types worsened with age. We found that loss of motor neurons could already be observed during the first larval stage (L1) post-hatching (Fig. 2H), and significantly increased in the following larval stage (L2) (data not shown) and in adults (Fig. 1B and Supplementary Material, Table S3). The accumulation of acquired fluorescence in the ventral cord neurons began to be visible after hatching (L1), and progressively increased with age (Fig. 4B). Thus, loss of motor neurons after smn-1 knock-down is a progressive phenomenon, a finding consistent with the progression of the disease.

The high level of conservation in the domain topology and sequence of SMN in C. elegans and humans (36% similarity) (18) prompted us to determine whether their function was also conserved. To address this point, we performed a rescue experiment in which the wild-type copy of the human SMN1 cDNA was expressed pan-neuronally (pun-119::hSMN1) in SMN-1 knocked-down animals. We observed a significant reduction in the number of dying neurons in these animals compared with wild-type animals (3.8 versus 5.6 fluorescent neurons per animal, P < 0.001) (Fig. 5A and Supplementary Material, Table S8), indicating a rescue of neuronal death using the human copy of the gene. We also found that the SMN1 human gene could rescue, at least partially, the phenotypes of reduced life span, thrashing, and backward movement, observed in the smn-1 null mutant k355 (Supplementary Material, Fig. S9A–D).

Taken together, these data support a significant functional conservation between the human and C. elegans genes, and confirm that the motor neuron death observed when smn-1 is knocked-down is the result of a specific reduction in the SMN1 protein.

smn-1 knock-down leads to the activation of the cell-death pathway

Having implied an apoptotic process in our findings, we next tested animals carrying mutations in genes of the canonical apoptotic pathway, including egl-1, ced-9, ced-4 and ced-3 (37–39), as well as cep-1, which encodes an ortholog of the human tumor suppressor p53 (40), and sir-2.1, a member of the Siruini family (41). The latter two genes act upstream of the apoptotic core machinery through two parallel mechanisms activated after DNA damage, and p53 has been demonstrated to interact with SMN1 in cell cultures (42). We transferred the smn-1 interfering construct at high concentration (HC) into six individual strains, each mutated in one of these genes. With the exception of cep-9, all the mutations were loss-of-function, to determine whether lack of their pro-apoptotic role prevented cell death. ced-9 is normally protective, and a gain-of-function mutation was used to determine a possible role in preventing smn-1-induced cell death. Using the acquired fluorescence in dying motor neurons as a readout, we observed a significant reduction in the number of fluorescent neurons in the smn-1 knocked-down strain (5.6 per animal) when in combination with a gain-of-function mutation in ced-9(n1950) (4.3 per animal, P < 0.001) or the loss-of-function mutations in ced-4(n1162) (2.8 per animal, P < 0.001), or ced-3(n717) (3.8 per animal, P < 0.001) (Fig. 5B). In contrast, loss-of-function mutations in cep-1(gk138), sir-2.1(ek394), and surprisingly also egl-1(n1084 n3082), did not significantly affect the number of dying motor neurons after smn-1 knock-down. When the smn-1 knock-down transgene was not in the background, we never observed fluorescent dying motor neurons in ced-9(n1950) gain-of-function mutants or ced-4(n1162) and ced-3(n717) loss-of-function mutants (Supplementary Material, Table S8). These results confirm that motor neurons undergo apoptotic cell death involving some of the key components of the classical pathway, and indicate that the accumulation of fluorescence in smn-1-silenced motor neurons is a late event in the degeneration process that partially occurs after the activation of the cell death pathway genes.

smn-1-induced degeneration is enhanced by mutations in Plastin3 and rescued by VPA

One of the main advantages of the cell-specific smn-1 knock-down animal models is that the use of different concentrations of the interfering construct makes it possible to mimic an allelic series with phenotypes of different severity. This facilitates the identification of genetic elements and pharmacological molecules that can either enhance or ameliorate the observed phenotypes. Plastin3 is an actin-bundling protein that has been reported to exert specific protective effects in SMA patients, as higher expression levels correlate with milder clinical symptoms (43). In addition, work in different species suggests that Plastin3 is a cross-species modifier of SMN function (43,44). Given that the C. elegans plst-1 gene encodes a protein similar to Plastin3 (56% identity and 73% similarity), we tested whether lack of plst-1 acts as a genetic enhancer of degeneration induced by smn-1 knock-down. When we introduced the plst-1(tm4255) loss-of-function mutation in the LC transgenic model, we observed that it significantly increased the number of dying motor neurons (from 1.6 to 3.8 dying neurons per animal, P < 0.001) (Fig. 5C). This result shows that Plastin3 plays a role in preventing neuronal death in vivo, and that smn-1 genetically interacts with Plastin3 in this process.

We next asked whether selected candidate drugs could rescue the degeneration phenotype. Resveratrol, VPA, 4-aminopyridine (4-AP) and gadoxadol (GH) have been shown to have a protective effect in different models of neuron degeneration (4,24,45). We found that, in the integrated strain of smn-1 knock-down, VPA significantly reduced the number of dying neurons (Fig. 5D). The extent of rescue was similar at 1 and 3 mM, but absent at 0.1 mM. These results demonstrate that VPA has a protective effect on neurons affected by smn-1 mutation.

Discussion

Although the genetic basis of SMA and the molecular functions of the SMN protein have been extensively studied, several fundamental questions remain largely unanswered (46). How does the absence of SMN1, a ubiquitous gene, induce the selective degeneration of motor neurons? Is the degeneration of motor neurons in SMA cell-autonomous or secondary to muscle defects? Do primarily developmental defects play a significant role in triggering the degeneration of neurons? The novel C. elegans model of SMA that we have presented here overcomes many of...
Degeneration culminates in neuronal death partially through an apoptotic mechanism

Motor neuron degeneration is the primary hallmark of SMA. The earliest morphological sign of degeneration that we observe is the loss of presynaptic densities: the gaps in the RAB-3 punctate pattern in the nerve cord are already present before the loss of visible GFP, and before the appearance of more severe signs of degeneration such as refractility of the cell body and positivity to cell death markers. Remarkably, neuronal death was not reported in a previous study in C. elegans of the null mutant smn-1(ok355) (20), and in our experiments with systemic smn-1 RNAi we observed neuronal loss at much lower penetrance than in smn-1(MNs RNAi) animals. These results can be explained by the hypothesis that maternal contribution largely prevents neuronal degeneration in the very few adult animals that survive past the early larval stages. This may be one of the reasons why neuron degeneration and death were not reported in previous studies using Drosophila melanogaster, zebrafish and C. elegans models of SMA (13,20,24,47).

SMN1-induced neuronal death could in principle occur through a necrotic or an apoptotic process. Our experiments conclusively demonstrate that the degeneration process in the smn-1(MNs RNAi) animals can activate an apoptosis-like cell death, whereas we never observed necrotic cells, which are easily distinguishable using DIC microscopy (data not shown). We have shown that the nuclei of dying neurons assume in some cases a button-like shape and become refractile and TUNEL-positive. Their cell bodies also bind TTR-52, a signal for engulfment that is pivotal for the clearance of dead neurons. Furthermore, we have identified part of the molecular pathway activated by smn-1 knock-down, revealing that it includes ced-9, ced-4 and ced-3 of the core apoptotic machinery, but not cep-1, sir-2.1 and egl-1. This result excludes the involvement of a DNA damage-induced apoptotic pathway (41). Our results reveal that neuronal death is independent of EGL-1 and that CED-9 has a minor role, confirming the findings of previous studies in which induction of apoptosis can occur downstream from, or in parallel with, egl-1 and ced-9 (48) (Supplementary Material, Fig. S10). Moreover, CED-9-dependent cell death pathways that are EGL-1-independent have already been reported in C. elegans (49). However, we never observed the complete suppression of the cell-death phenotype, even when apoptotic genes were absent. The presence of residual cell death even in ced-9, ced-4 and ced-3 mutants suggests that the apoptotic pathway is only one of the cell death mechanisms activated after smn-1 knock-down, and that it may require additional caspase or non-apoptotic/alternative death mechanisms (50,51). We therefore suggest that CED-4/Apaf-1 is also activated by an alternative upstream mechanism and that CED-4/Apaf-1 itself activates other downstream effectors, in addition to CED-3/ICE-caspase (Supplementary Material, Fig. S10). Indeed, CED-4-dependent cell death pathways that are CED-3-independent have already been reported in yeast strains, in which CED-4 is overexpressed (52), and in C. elegans (49–51), for example, after RNAi knock-down of the presumptive anti-apoptotic protein, ICD-1 (53). Moreover CoQ depletion triggers a cell death
mechanism in D-type motor neurons that is independent of EGL-1 and CED-9, but requires CED-4 and CED-3, with CED-4 also activating other downstream effectors in addition to CED-3 (54). Thus, the downstream genes alternative to CED-3 remains to be elucidated, although these may include other members of the caspase family or novel components.

Accumulation of GFP as a new read-out for dying neurons

Neuronal degeneration is detectable as the accumulation of GFP in dying motor neurons, an unexpected and novel phenotype whose specific dependence on the knock-down of smn-1 has been confirmed using cell-death markers and genetic mutants. GFP accumulation was a late event in neuronal degeneration, as (i) it occurred after loss of RAB-3 and after the activation of the cell death pathway genes, (ii) it was associated with indicators of cell death, and (iii) the number of GFP-positive neurons was very low in the early larval stages and strongly increased with age. We still do not know the mechanism by which GFP accumulates in dying motor neurons. It is possible that transcription from chs-2 or other promoters in dying motor neurons, where chs-2 is normally not active, is not tightly controlled such that small amounts of gfp transcripts are made and translated. An alternative mechanism is that GFP, expressed in the appropriate small amounts of chs-2, or other promoters in dying motor neurons, where chs-2 is normally not active, is not tightly controlled such that small amounts of gfp transcripts are made and translated. An alternative mechanism is that GFP, expressed in the appropriate
the accumulation of GFF is a specific, late sign of the degeneration of the motor neurons in which smn-1 has been knocked-down, and is never observed in negative controls. Further studies are needed to elucidate the mechanism of accumulation and whether or not this phenomenon can also be used as a marker of other neuronal apoptotic deaths.

**Plastin3 interaction with SMN1 and the axonal theory of SMA pathogenesis**

Plastin3, an actin-bundling protein, was the first documented modifier of SMA and a high level of its expression has been shown to have a protective effect in patients (43). Plastin3 has also been shown to be a cross-species modifier of SMN function. Overexpression of Plastin3 rescues the defects associated with SMN1 down-regulation in motor neurons of SMA mouse embryos and in a zebrafish model of SMA (43,55). Mutation of Fimbrin, the Drosophila ortholog of Plastin3, exacerbates DmSmn loss-of-function defects (44). When the C. elegans ortholog, plst-1, is knocked-down it causes unclear and opposite effects on growth defects and on pharyngeal pumping alterations observed in smn-1(ok355) mutants (44). We have now demonstrated that the loss of function of plst-1 enhances the degeneration and neuronal death of motor neurons in smn-1(MNs RNAi) animals, confirming a conserved neuronal protective role of this protein. The protective role of plst-1, together with the early loss of presynaptic densities in smn-1(MNs RNAi) animals, suggests that SMN1 loss-of-function in axons and at synaptic terminals could underscore the pathophysiology of SMA. It has been suggested that the specific association between SMN1 and a subset of mRNAs and proteins in the axonal compartment could be pivotal for the maintenance of axons and synapses in motor neurons. According to this hypothesis, known as the ‘axonal theory’ of SMA pathogenesis (56), this process could be selectively impaired in SMA, leading to the degeneration and loss of neurons in affected patients. The alterations at the presynaptic terminals may trigger a ‘die back’ mechanism that eventually results in neuronal death. These results suggest that the specific role of smn-1 in the presynaptic subcellular compartment requires a deeper analysis.

**Uses of the SMA model for pharmacological and genetic screenings**

Pharmacological screenings of SMA drugs, using cellular models and a few available animal models of SMA, including C. elegans (24), have already been performed with interesting results. However, in most of the existing animal models of degeneration and death of motor neurons either are not present or cannot be easily monitored in vivo (19,20). The novel, viable model of neuron degeneration that we describe is particularly suited for approaches aimed at identifying molecules specifically capable of protecting against neuronal degeneration and death. The small group of chemicals tested here was chosen for their ability to improve the deficits in locomotion and fitness caused by loss of function of smn-1 in C. elegans, or their proven protective role in other C. elegans models (4,24,45). We obtained a significant rescue with the anticonvulsant VPA, a deacetylase inhibitor. VPA has been associated with an increase in SMN genes expression in cell cultures and has been successfully used in mouse SMA models (4,46). Our results reveal that VPA can antagonize the neuronal death caused by smn-1 depletion in vivo, confirming the relevance of our model for drug screenings. The results of clinical trials investigating the potential of VPA as an SMA therapeutic have been inconsistent, demonstrating that it is only beneficial in a restricted subset of patients. Our results lend support to the use of VPA in clinical trials and our model could also play an important role in deciphering factors that influence the individual response to this drug. Interestingly, the two molecules, 4-AP and GH, that rescued the fitness defects in previous C. elegans smn-1 mutants (24), were not effective in our model, confirming that the cell-specific RNAi approach developed here can reveal new molecules that are specifically important for neuron degeneration and not for the general functions of smn-1.

There are two additional points worth mentioning regarding the value of the cell-specific knock-down of smn-1. First, this approach offers the opportunity to analyze the effects of smn-1 depletion in different neuronal sub-classes and/or in other non-neuronal tissues, with the goal of ultimately understanding the basis of the specificity of motor neuron loss observed in SMA patients. Second, by using different concentrations of the smn-1 interfering construct it is possible to modulate the extent of the cell-specific knock-down, and we have indeed obtained different lines presenting phenotypes of increasing severity, similar to a genetic allelic series. This has allowed us to test genetic mutations and drugs expected to suppress (e.g. ced-3, VPA) or worsen (e.g. plst-1) the phenotypes of smn-1(MNs RNAi) animals (Fig. 5B–D). Complementary models presenting different penetrance of the defects could be used to establish screens to identify genetic modifiers of smn-1 knock-down capable of either enhancing or suppressing neuronal degeneration and death. This could help to elucidate the molecular mechanisms underlying SMA and therefore its pathogenesis, and drive the discovery of new drug targets for this disease.

**Materials and Methods**

**Strains**

Nematodes were grown and handled following standard procedures, under uncrowded conditions, at 20 °C on NGM (nematode growth medium) agar plates seeded with Escherichia coli strain OP50 (57). Wild-type animals were C. elegans variety Bristol, strain N2. The alleles and transgenic strains used in this work were: unc-55(e1170) I, rrf-3(pk1426) II, smn-1(ok355) I, ced-4(n1162) III, sir-2.1(ok434) IV, ced-3(n171) V, cep-1(gk138) I, ced-1(e1735) I, ced-2(e1752) IV, oxtl2[punc-47:GFP; lin-15(+)+] X, juls76[punc-25:GFP; lin-15(+)+] II and wls48[punc-17:GFP] provided by the Caenorhabditis Genetics Center (CGC), which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440); Is[punc-47:RFP] kindly provided by K. Shen (Stanford University, USA); plst-1(tm4255) provided by S. Mitani, through the National Bio-Resource Project of the MEXT, Japan; smIs119[phsp-16.2::TTR-52::ChFP; KSHI(+)+] unc-76(+)+] kindly provided by D. Xue (University of Colorado, USA); egl-1(n1084n3082) and ced-9(n1950) kindly provided by M. Hengartner (University of Zurich, Switzerland). The transgenes obtained in this work were: gbEx29[punc-25::smn-1 RNAI HC], gbEx571[punc-25::smn-1 RNAI MC], gbEx502[punc-25::smn-1 RNAI LC], gbEx566[punc-25::kal-1 RNAI LC], gbEx62s[punc-25::kal-1 RNAI MC], gbEx543[punc-25::kal-1 RNAI HC], gbEx588[punc-25::unc-70 RNAI MC], gbEx529[punc-119::hSMN1], gbEx540[punc-119::DsRed2], gbEx580[punc-25::smn-1 RNAI HC], gbEx581[punc-25::smn-1 RNAI HC], gbEx612[pchs-2::GFP], gbIs4[punc-25::smn-1 RNAI HC],...
A full description of the transgenes is available in the Supplementary Material, Supplementary Methods section. Genetic crosses were made to transfer transgenes to the appropriate genetic background. In all cases, the presence of the mutant allele was verified by polymerase chain reaction (PCR) followed, when necessary, by sequencing. Primer sequences are available on request. Two independent clones with the same genotype were examined after each cross (see Supplementary Material, Supplementary Tables) and the mean of the two clones has been reported in the results.

Neuron-specific RNAi knock-down

D-type motor neuron-specific RNAi transgenes were constructed as previously described (25) by PCR-fusing a D-type motor neuron-specific promoter to an exon-rich region of the target gene (58). To knock-down a gene of interest we used a short form of unc-25/GAD promoter (180 bp), which is specifically expressed from embryonic to adult stages in the 19 GABAergic D-type motor neurons in the ventral cord and not in other GABAergic neurons (RMF, AVL, DVB and RJS) (59). We confirmed the expected expression pattern of this promoter using a gfp reporter approach, and successfully tested the ability of this promoter to knock-down a control gene, specifically in D-type motor neurons (data not shown). For the genes to be knocked down we amplified, from genomic DNA, the same exon-rich region that was used for the RNAi plasmid library, prepared for RNAi by feeding experiments (60). Exon-rich regions were amplified in two separate PCR reactions to obtain the sense and antisense fragments. The unc-25 promoter was amplified using specific primers. The promoter was subsequently fused to each orientation of the target gene by PCR fusion using internal primers, as previously described (25,58). Primer sequences are available on request.

Transgenic lines

GB300 [pchs-2::NLS::DsRed2] plasmid was obtained by removing the chs-2 promoter from GB301 [pchs-2::NLS::GFP] (33) and inserting it in front of the coding sequence of pVH14.05 (promoterless DsRed2 vector, kindly provided by H. Hutter, Simon Fraser University, Burnaby, Canada) using SalI and BamHI restriction sites. pSM punc-25::ChFP::RAB-3 was generated by removing the unc-25 promoter from pNV punc-25::GFP and inserting it in front of the coding sequence in the plasmid pNV ChFP::RAB-3, using FseI and Ascl restriction sites. Germline transformation was performed as described previously (61). Sense and antisense fusions to the GABAergic promoter were pooled into equimolar amounts and microinjected at different concentrations. HC, MC and LC of knocking-down constructs were injected, which corresponded to 200, 10 and 2 ng/µl, respectively. The LC knock-down construct was injected in rrf-3 (pk1426) mutants, which are more sensitive to neuronal RNAi, to enhance the knock-down effects (61). pSM punc-25::ChFP::RAB-3 plasmid was injected together with an empty pSM plasmid to obtain vdsEx410 [pSM punc-25::ChFP::RAB-3; pSM; pSM pdr-1::GFP]. vdsEx410 and gbEx29 transgenic lines were integrated by TMP/UV treatment to obtain vdsI4 and gbIs4, respectively. The co-injection markers used in this work were: pdr-1::GFP (kindly provided by C. Bargmann, The Rockefeller University, New York, USA; GFP expression in AWC and AWB neurons), pJM371 pelt-2::NLS::RFP and pJM67 pelt-2::NLS::GFP (kindly provided by J. McGhee, University of Calgary, Canada; RFP and GFP expression in the intestinal nuclei), GB301 pchs-2::NLS::GFP and GB300 pchs-2::NLS::DsRed2 (GFP and DsRed2 expression in 17 nuclei of the pharynx). The injection markers were co-injected at 10 to 30 ng/µl (see Supplementary Material, Supplementary Methods). At least two transgenic lines were examined for each experiment (see Supplementary Material, Supplementary Methods) and the mean of the lines has been reported in the figures. All transgenic strains are listed in the Supplementary Material, Supplementary Methods.

Behavioral assay

Well-fed, young adult animals were used for backward movement assay to test D-type motor neurons function. The assay was performed blind on NGM plates, 6 cm in diameter, seeded with bacteria. Using an eyelash the animal was touched first on the tail to induce a forward movement and then on the head to test for backward movement. Defective movement was scored when animals were unable to fully move backward. For each data set, the percentage of defective animals among the total number of tested animals was calculated.

TUNEL assay

Young adult and adult animals were fixed and permeabilized as previously described for GABA neurotransmitter visualization (62). Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) reactions were then performed by using an In Situ Cell Death Detection Kit, POD (Roche Diagnostics). Briefly, permeabilized animals were washed twice with PBS and incubated with 50 µl of TUNEL reaction mixture for 60 min at 37°C. For visualization of samples under a light microscope, the animals were washed three times with PBS before adding 50 µl of Converter-POD. The animals were incubated for 30 min at 37°C and then rinsed three times with PBS. Finally, for signal conversion, 100 µl of DAB substrate (Sigma-Aldrich) was added to each sample, followed by incubation at room temperature for 10 min. Animals were washed three times with PBS before mounting on slides and analyzing under a light microscope.

Microscopy analysis

Animals were immobilized in 0.01% tetramisole hydrochloride (Sigma-Aldrich) on 4% agar pads and visualized using Zeiss Axioskop or Leica DMi6000B microscopes. All microscopes were equipped with epifluorescence and DIC Nomarski optics and images were collected with an Axioscam digital camera or with Leica digital cameras DFC 480 and 420 RGB. To discriminate dyeing motor neuron fluorescence from endogenous autofluorescence, we used either a Zeiss filter set 09 (that allowed observation of intestinal cell autofluorescence in yellow and GFP-positive dying cells in green) on a Zeiss Axioskop microscope or a Leica TCS SP2 AOBS laser scanning confocal microscope.

Rescue experiments

The rescue construct for pan-neuronal expression of hSMN1 was created by PCR-fusion (58) of three fragments: the promoter of unc-119 gene, the cDNA of the human SMN1 gene and the 3’ untranslated region (UTR) of unc-54. The unc-119 promoter has been previously used for neuronal-directed rescue of smn-1 (20) and is considered to be a strong transcriptional inducer (63).
The human SMN1 gene was amplified from plasmid pcDNA4::hSMN1. To increase the stability of the rescuing construct, we added the 3’ UTR of the unc-54 gene (which was amplified from plasmid pPD95.75, kindly provided by A. Fire, Stanford University, Stanford, USA) to the 3’ end of the human cDNA. This construct was microinjected at two concentrations (20 and 100 ng/μl) to find the best conditions and first injected in wild-type animals to exclude non-specific effects due to overexpression of the gene (data not shown). The same transgenic array was then transferred to animals in which smn-1 was knocked-down in the motor neurons. As a negative control the human SMN1 gene was replaced with DsRed2 (amplified from plasmid pVH14.05), a gene not present in the C. elegans genome. The specificity of the rescue with the human gene was confirmed by the lack of rescue when DsRed2 was expressed in smn-1 knocked-down animals, using the same experimental conditions (Fig. 5A and Supplementary Material, Table S8).

Drug treatments

Individual compounds (Sigma Aldrich) were dissolved in dimethylsulfoxide (Sigma Aldrich) or H2O and then added to molten NGM agar to the desired concentration before pouring into petri dishes. Control NGM plates contained the appropriate dilution of vehicle. Plates were allowed to dry for 1 day before seeding with OP50 bacteria, after which they were incubated at room temperature. Gravid adults were then transferred onto the drug/control plates to lay eggs and removed after 24 h. Each experimental condition was run in blind duplicate or triplicate. For drug treatments, the integrated line with an HC of interfering construct (HCl) was used so that all animals tested were affected due to the loss of incomplete segregation of the transgene that is obtained following integration.

Statistical analysis

Primer of Biostatistics and GraphPad Prism softwares were used for statistical analyses. The statistical significance was determined using z-statistic tests, unpaired t-test or Mann–Whitney test, as indicated, comparing each sample against the control. The standard error of the mean was used to estimate variation within a single population.

Supplementary Material

Supplementary Material is available at HMG online.

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