**Introduction**

Motoneuron diseases (MNDs) constitute a group of devastating neurodegenerative disorders characterized by the progressive and selective degeneration of motoneurons in the brain and/or spinal cord. Amongst MNDs, spinal muscular atrophy (SMA) and amyotrophic lateral sclerosis (ALS) are the most common in children and adults, respectively. SMA is an inherited monogenic disease caused by loss-of-function mutations in the SMN1 gene and characterized by selective proximal muscle weakness and atrophy (1, 2). ALS is an inherited heterogeneous disease with various types (type 0-IV), which are classified according to the age of onset and severity of symptoms. By contrast, ALS is inherited in approximately 10% of cases. With over 30 genes described to date, ALS-causing mutations are most frequently found in chromosome 9 open reading frame 72, superoxide dismutase-1 (SOD1), fused in sarcoma and TAR DNA binding protein genes (3). Familial cases are clinically indistinguishable from sporadic cases, both of which display upper and lower motoneuron signs as a common feature, which also illustrates the biological complexity of the disease.

As SMA and ALS are genetically distinct, they have traditionally been considered as separate disorders (3, 4). However, accumulating evidence suggests that the convergence of aberrant pathways underpins the selective vulnerability of motoneurons in the context of ubiquitously expressed mutant proteins. Indeed, SMA and ALS...
share pathological features such as excitability defects in motoneurons that implicate intrinsic and extrinsic determinants, differential sprouting ability that underlie the selective vulnerability of motoneuron populations, non-cell-autonomous mechanisms where astrocytes and microglial cells produce neuroinflammatory and deleterious factors including tumor necrosis factor alpha, nitric oxide and interleukin-1β (5). More recently, a transcriptomic study conducted on vulnerable motoneurons isolated from the spinal cord of the Smmn"∆2-" mouse model of SMA (6), has revealed a significant upregulation of the Fas (CD95) death receptor (7). Furthermore, motoneurons derived from SMA patient-induced pluripotent stem cells, which degenerate over time in culture, show upregulation of Fas ligand (FasL) and activation of downstream caspases (8). Interestingly, we have previously demonstrated that Fas triggers a motoneuron-restricted death pathway that is exacerbated by ALS-linked mutant SOD1 (9-11). The underlying pathway implicates the activation of p38 kinase and caspases as well as the upregulation of collapsin response mediator protein 4 (CRMP4), a protein implicated in axonal degeneration (12). Thus, the increased death activity of Fas in SMA and ALS could be a novel link between these two genetically distinct motoneuron diseases.

Here, we initially investigated whether Fas signaling was involved in the death of SMA motoneurons. We show that a population of primary motoneurons purified from Smmn"∆2-" mice progressively degenerate in culture. We observed the selective death of proximal Smmn"∆2-" motoneurons that can be blocked by interfering with the endogenous Fas-FasL interaction, activation of caspases, p38 kinase, Rho-associated kinase (ROCK) and CRMP4 up-regulation. Unexpectedly, we observed that the application of exogenous soluble FasL also induces axonal growth in both wildtype and SMA motoneurons. Fas-dependent axon extension implicates extracellular signal-related protein kinase (ERK) and caspases.

Materials and Methods

Animals

All animal experiments were approved by the national ethics committee on animal experimentation and were performed in compliance with the European community and national directives for the care and use of laboratory animals. Smmn"∆2-" and Smmn"−/−" mice were maintained on a C57BL/6J background under specific pathogen-free conditions. Smmn"∆2-" and Smmn"−/−" were bred with healthy controls and Smmn"+/+" mice to generate progeny with the Smmn"∆2-" healthy controls and Smmn"−/−" SMA mice (6).

Reagents

Soluble recombinant human FasL, enhancer for ligand and recombinant human Fas-Fc were purchased from Enzo Life Sciences. z-IETD-fmk, z-DEVD-fmk, Ac-LEHD-ckmk, SB203580, PD98059 and Y-27632 were purchased from Merck. Rabbit polyclonal antibodies against Forkhead box P1 (FoxP1) (ab16645) and CRMP4 (ab101009) were purchased from Abcam. Monoclonal antibodies against Islet-1 and anti-Islet-2 (4D5 and 2D6) were from the Developmental Studies Hybridoma Bank of Iowa University. Rabbit polyclonal antibodies against β-tubulin III (T2200) was from Sigma-Aldrich. Chicken polyclonal antibodies against GFP (ab13970) were purchased from Abcam.

Motoneuron culture

Mice at embryonic day (E)12.5 were kept on ice in Hibernate E (ThermoFisher Scientific) and the genotype was determined by PCR of tail DNA (13). Motoneurons were isolated from the spinal cord of Smmn"∆2-" and Smmn"−/−" embryos using 5.2% iodixanol density gradient centrifugation combined with p75-based magnetic cell isolation (Miltenyi Biotec) as we previously described (14). Motoneurons from the spinal cords of Hb9::GFP embryos were isolated using iodixanol density gradient centrifugation. Motoneurons were plated on poly-ornithine/laminin-treated wells in the presence of a cocktail of neurotrophic factors (0.1 ng/ml glial-derived neurotrophic factor, 1 ng/ml brain-derived neurotrophic factor and 10 ng/ml ciliary neurotrophic factor in supplemented Neurobasal medium (ThermoFisher Scientific)). Supplemented Neurobasal medium contains 2% (vol/vol) horse serum, 25 µM L-glutamate, 25 µM β-mercaptoethanol, 0.5 µM L-glutamine, 2% (vol/vol) B-27 supplement (ThermoFisher Scientific) and 0.5% penicillin/streptomycin.

Immunocytochemistry

Motoneurons were plated on poly-ornithine/laminin-treated glass coverslips at the density of 5000 cells per cm². Neurons were incubated for 15 min on ice in 2% paraformaldehyde (PFA) in phosphate-buffered saline (PBS)-culture medium (1:1) and for an additional 15 min in 4% PFA in PBS. Cells were washed with PBS and incubated for 1 h at room temperature in PBS containing 4% BSA, 4% donkey serum and 0.1% Triton X-100. Cells were incubated overnight at +4°C with the primary antibodies diluted in PBS containing 4% BSA, 4% donkey serum and 0.1% Triton X-100. After PBS washes, cells were incubated with fluorophore-conjugated secondary antibodies (ThermoFisher Scientific) for 1 h at room temperature. Cells were then washed in PBS and incubated for 5 min in PBS containing 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). Coverslips were mounted onto glass slides using Mowiol medium.

Production of recombinant adeno-associated virus

Adeno-associated-viral (AAV) vectors containing short hairpin RNA (shRNA) targeting Crmp4 or control mismatch were previously developed and validated (12). The shRNA stem sequence used were shCrmp4, 5'-GAACCTGTATATGCCTGA-3' and shCrmp4-mismatch, 5'-GAACCTGTATATCTCTGA-3'. These shRNAs were cloned into a bipartite pAAV vector with shRNA expression controlled by the H1 promoter and EGFP expression controlled by the cytomegalovirus promoter. AAV2/6 vectors were produced by the Bertarelli platform for Gene Therapy at EPFL (Lausanne, Switzerland). Briefly, pAAV plasmids were co-transfected with a pDP6 helper plasmid into HEK293-AAV cells (Agilent). Cells were lysed 72 h after transfection and viral particles were purified using iodixanol gradient followed by separation ion-exchange chromatography (GE Healthcare). The infectivity titer of each virus (expressed as transduced units/ml) was determined following infection of HEK293T cells by real-time PCR using primers for beta-globin intron and human albumin.

Motoneuron survival and axon length

Motoneurons were seeded at a density of 750 cells/
cm². Three hours (0 DIV), 48 h (2 DIV) or 72 h (3 DIV) after plating, the number of surviving motoneurons was determined by counting two diameters. Between 60 and 150 motoneurons were counted in 2-cm diameters of each well by fluorescence (Hb9::GFP motoneurons) or phase contrast microscopy (p75-immunopurified SMA motoneurons)(10). To compare values between different experiments, survival is expressed relative to the control condition where motoneurons are cultured only in the presence of the cocktail of neurotrophic factors. For the quantification of axon length, motoneurons were seeded at a density of 750 cells/cm². Motoneurons were then processed for immunostaining with anti-GFP (for Hb9::GFP motoneurons) or β-tubulin III (for Smn2B-/-; motoneurons) antibodies. Images were acquired with a ZEISS Axio Imager Z2 Apotome and axon length was determined using the ImageJ software and NeuronJ plugin (National Institutes of Health, USA) in 60-130 neurons per condition from triplicate wells per experiment (15). Total axon length was determined by measuring the length of the longest neurite with connected branches of GFP-positive or β-tubulin III-positive motoneurons.

Statistical analyses

Statistical significance was determined by unpaired two-tailed t-test, one-way or two-way analysis of variance (ANOVA) followed by a Tukey's post hoc test. Statistical analyses were done with Graphpad Prism software (GraphPad Software). Significance was accepted at the level of P < 0.05.

Results

Selective death of Smn-depleted proximal motoneurons in vitro

To study how Smn depletion impacts motoneuron death, we first isolated spinal motoneurons from Smn2B-/-, Smn2B-/-; and wildtype embryos and determined their survival over time in culture. While the survival of Smn2B-/-; motoneurons is comparable to that of wildtype and remains stable over time, a notable decrease in the survival of Smn2B-/-; motoneurons can be observed 2 and 3 days after seeding (Figure 1A). Thus, motoneurons from SMA mice show an altered survival in vitro, although the survival of about 60% of them is not affected by Smn depletion.

Distal limb muscles are less affected than proximal muscles in SMA (16-18). FoxP1 transcription factor is selectively expressed by lateral motor column (LMC) neurons that innervate distal limb muscles (19). We asked whether LMC motoneurons have a differential susceptibility to Smn depletion. We immunostained Smn+/+ and Smn2B-/-; motoneurons for FoxP1 and the LIM homeodomain transcription factors Islet-1 and Islet-2 that are expressed by all somatic motoneurons and determined the relative proportion of LMC neurons after 3 days in vitro (DIV) (Figure 1B). We observed that the proportion of FoxP1+ motoneurons was unchanged between the time of seeding and 3 DIV in control Smn2B-/-; mice. As expected, the survival of Islet-1/-2 Smn2B-/-; motoneurons was significantly decreased at 3 DIV compared to seeding time. However, the relative proportion of FoxP1+ Smn2B-/-; motoneurons remains unchanged between the time of seeding and 3 DIV (Figure 1B), suggesting an increased vulnerability to Smn depletion among the population of Islet-1/-2 FoxP1+ proximal motoneurons.

Death of SMA motoneurons involves the motoneuron-selective Fas death signaling

Since Fas is upregulated in vulnerable spinal motoneurons of Smn2B-/- mice and in somatic motoneurons of delta 7 SMA mice (7, 20), and has also been proposed to induce death of human motoneurons derived from SMA induced pluripotent stem cells (8), we investigated whether Fas might trigger the death of embryonic Smn2B-/-; motoneurons. When we blocked endogenous Fas-FasL interaction by the Fas-Fc antagonist (11), we prevented motoneuron loss induced by Smn depletion (Figure 2A). It is noteworthy, that when Fas was exogenously activated by the soluble recombinant form of FasL (sFasL), the proportion of Smn2B-/-; motoneurons that die after 2 DIV was not increased (Figure 2A). These results indicate that Fas triggers the death of motoneurons in the same proportion as Smn depletion. To evaluate whether the increased vulnerability of the FoxP1+ population of Smn2B-/-; motoneurons is an idiosyncrasy of Smn loss (Figure 1C), we treated wildtype motoneurons with sFasL and determined the percentage of death induced by Fas in FoxP1+ and Foxp1-/- motoneuron populations. We did not observe any different vulnerability to Fas activation between the distal and proximal populations of wildtype motoneurons (Figure 2B).

We previously demonstrated that Fas-induced death of motoneurons involves the FADD-Caspase-8 pathway that synergistically acts with a Daxx-p38 kinase pathway (10). To test whether Fas signaling involves the classical caspase-8, -9 and -3 caspases in Smn2B-/-; motoneuron death, neurons were treated with their respective inhibitors. We observed that the death of SMA motoneurons was completely blocked by the z-IETD-fmk caspase-8 inhibitor, Ac-LEHD-cmk caspase-9 inhibitor and the z-DEVD-fmk caspase-3 inhibitor (Figure 2C). We then inhibited p38 kinase activity using SB203580, which completely prevented Smn-dependent death of motoneurons (Figure 2C).

Figure 1. Selective death of Smn2B-/- motoneurons in vitro. (A) Survival of motoneurons immunopurified from wildtype (Smn+/+), Smn2B-/-; and Smn2B-/-; embryonic spinal cord. Motoneurons were cultured in the presence of neurotrophic factors and the percentage of surviving neurons was determined at the indicated time. For each genotype, the survival is expressed relative to the survival 3 hours after plating (0 DIV). (B) Smn2B-/-; and Smn2B-/-; motoneurons were cultured for 0 and 3 DIV and immunostained with anti-Islet-1/-2 and FoxP1 antibodies. The proportion of FoxP1+ motoneurons is expressed as the percentage of the number of Islet-1/-2+ neurons at 0 DIV (time of seeding) for each genotype. Values are means ± standard error of the mean (SEM), ***P < 0.01, ****P < 0.001, n.s, non-significant, 1-way (B) or 2-way (A) ANOVA with Tukey's post hoc test. Data are representative of 3-4 independent experiments, each done in triplicate. Statistical attributes are shown for Smn+/+ vs Smn2B-/-; Smn2B-/-; vs Smn2B-/-; at 2 DIV, ***P < 0.01 and Smn2B-/-; vs Smn2B-/-; at 3 DIV, ****P < 0.001. Smn+/+ and Smn2B-/-; are statistically indistinguishable at all times of culture.
explored the functional implication of CRMP4 in the death of SMA motoneurons. We first determined CRMP4 levels in vivo. Consistently, the protective effect of Fas-Fc on SMA motoneuron survival was determined after 2 DIV and expressed relative to the number of motoneurons at the time of plating (0 DIV) in each condition. (B) Motoneurons from E12.5 spinal cord of Hbb::GFP embryos were cultured for 24 h and then treated (or not) with sFasL. The percentage of GFP FoxP1- and GFP FoxP1+ motoneurons killed by Fas was determined 48 h later. (C) Smn2B+ motoneurons were cultured in the presence of Fas-Fc (1 µg/ml), z-IETD-fmk (10 µM), Ac-LEHD-cmk (1 µM), z-DEVD-fmk (10 µM) or SB203580 (10 µM). Motoneuron survival was determined after 3 DIV and expressed relative to the number of motoneurons at the time of plating (0 DIV, none). (D) Smn2B- and Smn2B+ motoneurons were cultured for 24 h and immunostained with anti-Islet-1/-2 and CRMP4 antibodies. Representative image, scale bar, 10 µm. (E) For the quantification of CRMP4 immunostaining, the CRMP4 mean fluorescence is expressed relative to Islet-1/-2 fluorescence (a.u, arbitrary unit). A total of 90 motoneurons were quantified by condition (n = 3). (F) SMA motoneurons were infected with 10 TU per cell of AAV-shCrmp4 and AAV-shCrmp4mis after plating. The survival of motoneurons was determined at 3 DIV and expressed relative to the control condition (none) at the time of seeding (0 DIV). (G) Smn2B- motoneurons were incubated (or not) with 10 µM of Y-27632 and 10 µM of PD98059 for 3 days. Motoneuron survival was expressed relative to the control condition at 0 DIV. Values are means ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001, n.s, non-significant, ANOVA with Tukey’s post hoc test (a,c,f,g) or t-test (b,e). Data are representative of at least three independent experiments, each done in triplicate.

Consistently, the protective effect of Fas-Fc on SMA motoneurons was also observed after 3 DIV.

Another signature of the motoneuron-specific Fas signaling is the upregulation of CRMP4, which we showed to contribute to neuronal degeneration both in vitro and in vivo. To investigate whether the death of SMA motoneurons involves CRMP4, we first determined CRMP4 levels in Smn2B- motoneurons. Quantification of fluorescence intensity showed increased levels of CRMP4 in Smn2B- compared to Smn2B+ motoneurons (Figure 2D,E). We then explored the functional implication of CRMP4 in the death of SMA motoneurons. We have previously shown that silencing of CRMP4, through AAV6 vectors encoding a shRNA against Crmp4 (AAV-shCrmp4) that can transduce up to 90% of motoneurons in vitro, prevents death of motoneurons induced by the Fas pathway. Reduction of CRMP4 expression in Smn2B- through AAV-shCrmp4 protected motoneurons from death induced by Smn depletion. Expression of a mismatch control (AAV-shCrmp4mis) had no effect on the survival of SMA motoneurons (Figure 2F). These results indicate that Smn depletion activates components of the motoneuron-restricted Fas death signaling.

The intracellular pathways underlying motoneuron degeneration under conditions of Smn depletion remain poorly understood. A combination of Fasudil that inhibits ROCK and selumetinib that inhibits ERK activity in SMA mouse neonates suggested that a ROCK to ERK cross-talk contributes to SMA pathogenesis. We then asked whether ROCK and ERK pathways were implicated in the death of SMA primary motoneurons. While inhibition of the ROCK pathway by Y-27632 prevented the death of motoneurons isolated from Smn2B-mice, inhibition of the ERK pathway by PD98059 had no effect on their survival (Figure 2G).

Exogenous Fas activation elicits axon outgrowth in motoneurons

The complexity of Fas signaling pathways is illustrated by its ability to promote neurite outgrowth in sensory neurons or increase the branching number in hippocampal neurons. These neuronal types are however resistant to Fas-mediated apoptosis. We previously demonstrated that activation of the lymphotoxin beta receptor (LT-βR) by its ligand LIGHT can elicit opposite responses in motoneurons by inducing either death or axon outgrowth. We then examined the effect of Fas engagement by sFasL on axonal growth. The Fas-mediated death of motoneurons requires at least 2 DIV. This observation is consistent with our finding that Smn2B- motoneuron death is not observed before 2 DIV (Figure 1A). Total axon length was thus determined following 24 h of treatment with sFasL. We observed that Fas activation significantly increases axon length in motoneurons (Figure 3A,B). We then asked whether this dual response to Fas stimulation could be impacted by Smn depletion. We found that exogenous Fas activation also promotes axonal elongation in Smn2B+ motoneurons (Figure 3C).

ERK, ROCK and caspase signaling are required for Fas-induced axon outgrowth

We previously showed that LT-βR activation in motoneurons elicits the ERK signaling pathway required for neurite outgrowth, but not cell death. To evaluate the relative contribution of the ERK pathway in Fas-induced axon outgrowth in motoneurons, we pharmacologically inhibited ERK with PD98059 following sFasL treatment. This reduced the total axon length of motoneurons following Fas activation (Figure 4A). However, pharmacological inhibition of the ERK pathway had no effect on the Fas-dependent death of motoneurons (Figure 4B). The serine/threonine kinase ROCK regulates actin cytoskeleton dynamics and prevents axonal growth. As shown above, the death of Smn2B- motoneurons involves ROCK signaling (Figure 2G). We, therefore, asked whether ROCK inhibition might impact axon length as well as the death...
of wildtype motoneurons following exogenous Fas activation. The addition of Y-27632 to motoneuron cultures treated with sFasL abolished Fas-induced axonal elongation (Figure 4C), as well as Fas-induced death of motoneurons (Figure 4D). This observation is consistent with the role of ROCK in Fas clustering (see Discussion).

Accumulating evidence shows that the function of executioner caspasases is not limited to apoptotic activity but may also be involved in neurite growth as well as axonal guidance (26). We have previously shown that z-DEVD-fmk caspase-3 inhibitor saves motoneuron from Fas-induced death (11). We also observed that Ac-LEHD-cmk caspase-9 inhibitor saves motoneuron from Fas-induced death (Figure 4E). We then applied Ac-LEHD-cmk and z-DEVD-fmk to assess the contribution of executioner caspase in Fas-induced neurite outgrowth. Total axonal length of motoneurons was significantly reduced when inhibitor of caspase-9 and caspase-3 were added with sFasL (Figure 4F). Combined, our results suggest that both the ERK and ROCK signaling pathways modulate the effect of Fas on axonal outgrowth but that only the latter contribute to the Fas-dependent death of motoneurons. Caspasases also seem to contribute to Fas-mediated axonal growth.

**Discussion**

The molecular mechanisms that drive motoneuron death in SMA remain elusive, and those, like the p53 pathway, which have been proposed as important are still the subject of conflicting results and debate (27-30). To explore SMA-related death mechanisms, we used the Smn2B/2B mouse model of SMA. Smn2B/2B mice harbor on one allele a three-nucleotide substitution mutation (termed 2B) within the exon splicing enhancer of Smn exon 7 and a null allele of Smn (13). Smn2B/2B mice show reduced Smn levels in the brain and spinal cord to approximately 20% of control levels (31), and displays the pathological hallmarks of SMA observed in patients such as muscle weakness and gait abnormalities. These SMA mice have a median lifespan of 30 days and are characterized by motoneuron loss and neuromuscular defects (6). We have shown here that motoneurons derived from Smn2B/2B mouse embryos have survival that is progressively reduced with time in culture. This loss of motoneurons is dependent on the Fas-FasL interaction and preferentially involves the subpopulation that innervates proximal muscles, based on FoxP1 expression. This selective vulnerability of FoxP1’ proximal SMA motoneurons is consistent with the selective proximal distribution of muscle weakness observed in SMA patients and mice (16, 32). The absence of FoxP1 expression, which we have used here for its role in defining the columnar identity of motoneurons (19), is also intriguing for other functional aspects related to a potential vulnerability to death. Indeed, levels of FoxP1 are decreased in the striatum of mice and patients with Huntington’s disease, and the forced expression of FoxP1 rescued cortical neurons from mutant Huntingtin-mediated toxicity (33). FoxP1 overexpression represses expression of proapoptotic genes in B cells (34). FasL was identified as one of these proapoptotic genes directly repressed by FoxP1 (35). Transcriptome analysis of a FoxP1-depleted human colon carcinoma cell line showed an upregulation of Forkhead box transcription factor class O (FOXO) target genes that include Fas (36). If we refocus on the spinal motor system, FasL is a downstream target of Foxxo3a to drive motoneuron death through Fas activation following neurotrophic factor deprivation (37). Here, we observed that wildtype FoxP1 and FoxP1’ motoneuron display similar susceptibility to Fas-induced death. Identity determinants,
by engaging essential factors for neuronal diversification, projection and connectivity patterns, may also contribute, alongside interactions with genes such as Smn, to provide a cell-intrinsic program of vulnerability.

Death of Smn-depleted motoneurons can be prevented by ROCK, but not ERK, inhibitor. Studies on the contribution of ROCK in SMA mice have led to diverse conclusions (22, 38, 39). This is likely related to the multi-systemic nature of the disease where various cell types contribute to the symptoms and may be the target of the inhibitors (40). The cellular modalities of ROCK and ERK inhibition (as well as culture conditions) may also help to explain the differences in ERK inhibitor effects between our observations made on immunopurified motoneurons and those made on mixed cultures of spinal cord obtained from the severe Taiwanese mice (22). On the contribution of the ROCK pathway in Fas-dependent death of SMA motoneurons, our observation is reminiscent of what was observed in Jurkat cells where ROCK inhibition by Y-27632 reduced apoptosis induced by Fas stimulation or what was reported in human dermal microvascular endothelial cells where Fasudil, another ROCK inhibitor, protected from Fas-induced death (41). In the Jurkat cells, it was shown that upon Fas activation ROCK phosphorylates ezrin and moesin, thus linking Fas to the actin cytoskeleton and facilitating Fas clustering and formation of the death-inducing signaling complex (42). Another evidence shows that the clustering of Fas, which occurs concomitantly with F-actin polarization and independently of the apoptotic program, requires ROCK activity (43). Even more interesting is that Fas activation in Jurkat cells induces RhoA which acts upstream of ROCK. Our data support the growing interest in ROCK as a therapeutic target for motoneuron diseases, including ALS, in which we and others have shown the role of Fas (44). Of note, Fasudil is already being evaluated in a double-blind, randomized, placebo-controlled phase Ila trial in patients with ALS (45). However, by acting very upstream in Fas signaling and abrogating therefore both deleterious (apoptosis) but also beneficial (axonal outgrowth) processes, the outcome of ROCK inhibition in vivo might also be interpreted under the light of Fas signaling.

Here, we report that Fas activation by the soluble form of FasL ligand can also elicit axon outgrowth in both wild-type and Smn<sup>2B/−</sup> motoneurons. Previously, Fas stimulation was shown to increase the number of branching points, but not neurite length, of hippocampal neurons, independently of caspase activation (24). Another study also reported that Fas activation induced neurite outgrowth in sensory neurons in a caspase-8-independent, ERK pathway-dependent manner (23). We here reproduce this feature of dual signaling of a death receptor in motoneurons by highlighting that Fas requires ERK and caspases to elicit axon outgrowth, while it requires caspases, but not ERK, to trigger death. While the ERK pathway, which can modulate the local translation of proteins, is widely recognized as a key promoter of neurite outgrowth (46), and localized caspase activity has been well documented during axon degeneration (47), the contribution of caspases to axonal elongation remains however elusive. The diversity of substrates of caspases already underlines the extent of their nonapoptotic function. Indeed, very recently, N-Terminomics has identified 906 caspase-3 and 124 caspase-9 substrates in healthy Jurkat cells (48). The majority were previously identified as apoptotic substrates, but other substrates were implicated in different pathways such as mRNA splicing, RNA metabolism, Notch-HLH pathway SUMOylation, HIV infection for caspase-3 and mRNA splicing, RNA metabolism, mitotic prometaphase, Rho GTPase signaling, and membrane trafficking for caspase-9. Another study in Jurkat cells following TRAIL activation, identified as caspase-3, -7, -8 substrates, proteins involved in endocytosis and vesicle trafficking (49). Additional caspase-3 substrate databases (MEROPS (50), CASBAH (51), and DegraBase (52)) have been described that illustrate the complexity of apoptotic and nonapoptotic caspase signaling. In Xenopus, it was shown that the chemotropic cue netrin-1 induces retinal growth cone attraction, requires caspase-3 activity which is downstream of p38 kinase (53). In chick embryos, caspase-3 is involved in the arborization pattern of developing axons of the ciliary ganglion (54). In the rat, growth cone formation following axotomy of sensory and retinal axons implicates p38 kinase and caspase-3 activity (55). In mouse hippocampal neurons, the clustering of the neural cell adhesion molecule at the growth cone induces caspase-8 and -3 activation, which is required for neurite outgrowth likely through local proteolysis of the spectrin meshwork (56). Thus, it will be imperative for future studies to examine the intracellular cascade that coordinates local protein translation, membrane remodeling and cytoskeleton dynamics in response to death receptor activation in motoneurons.

Our study reveals a common death mechanism that underpins the vulnerability of motoneurons to ALS- and SMA-causing genetic determinants. The intracellular relays of the motoneuron-restricted Fas death pathway are also recruited to execute the death of SMA motoneurons. These common signaling intermediates may therefore appear as therapeutic targets for SMA. However, the duality of motoneuron response to Fas activation provides an important insight into the therapeutic approaches to be considered.

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Conflict of interest
The authors declare that there is no conflict of interest.

Author contribution
SB, MB and CR conceived and designed the analysis; SB and CR collected the data; SB, RY, DC, NBM, CR performed the experiments; RK and BS provided Smn mice and viral vectors respectively; SB, MB, RK, CH, FS, BS and CR analyzed the results; CR administrated the project, CR and SB drafted the manuscript and all authors participated in revisions. All authors reviewed the results and ap-
proved the final version of the manuscript.

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