Chondrolectin affects cell survival and neuronal outgrowth in *in vitro* and *in vivo* models of spinal muscular atrophy

James N. Sleigh¹, Antón Barreiro-Iglesias²,†, Peter L. Oliver¹,†, Angeliki Biba¹, Thomas Becker², Kay E. Davies¹, Catherina G. Becker² and Kevin Talbot¹,3,*

¹Department of Physiology, Anatomy and Genetics, MRC Functional Genomics Unit, University of Oxford, South Parks Road, Oxford OX1 3PT, UK, ²Centre for Neuroregeneration, University of Edinburgh, Edinburgh EH16 4SB, UK and ³Nuffield Department of Clinical Neurosciences, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU, UK

Received July 15, 2013; Revised September 2, 2013; Accepted September 20, 2013

Spinal muscular atrophy (SMA) is characterized by the selective loss of spinal motor neurons owing to reduced levels of survival motor neuron (Smn) protein. In addition to its well-established role in assembling constituents of the spliceosome, diverse cellular functions have been proposed for Smn, but the reason why low levels of this widely expressed protein result in selective motor neuron pathology is still debated. In longitudinal studies of exon-level changes in SMA mouse model tissues, designed to determine the contribution of splicing dysfunction to the disease, we have previously shown that a generalized defect in splicing is unlikely to play a causative role in SMA. Nevertheless, we identified a small subset of genes that were alternatively spliced in the spinal cord compared with control mice before symptom onset, indicating a possible mechanistic role in disease. Here, we have performed functional studies of one of these genes, chondrolectin (*Chodl*), known to be highly expressed in motor neurons and important for correct motor axon outgrowth in zebrafish. Using *in vitro* and *in vivo* models of SMA, we demonstrate altered expression of *Chodl* in SMA mouse spinal motor neurons, show that *Chodl* has distinct effects on cell survival and neurite outgrowth and that increasing the expression of *chodl* can rescue motor neuron outgrowth defects in Smn-depleted zebrafish. Our findings thus link the dysregulation of *Chodl* to the pathophysiology of motor neuron degeneration in SMA.

**INTRODUCTION**

Spinal muscular atrophy (SMA) is a currently incurable autosomal recessive motor neuron disorder affecting ~1 in 10,000 newborns (1,2), in which reduced levels of the widely and constitutively expressed survival motor neuron (Smn) protein lead to specific degeneration of spinal cord motor neurons, denervation and muscle atrophy (3). Two genes encode Smn. The ancestral gene, *Smn1*, homozgyously deleted or mutated in SMA patients, produces sufficient Smn protein for normal cellular function, whereas its parologue, *Smn2*, produces drastically reduced amounts of full-length Smn protein because alternative splicing removes the critically important exon 7 in ~90% of transcripts (4). *Smn2* copy number can modify the disease phenotype, with increasing amounts of full-length Smn protein resulting in the clinically observed spectrum of disease from type I (severe infantile onset) to type IV (milder adult onset) (5,6). Smn has been suggested to play a role in a number of fundamental cellular processes including axonal transport of mRNA and ribonucleoproteins (7–9), the regulation of transcription (10) and stem cell maintenance (11). However, the critical role of the Smn complex in the assembly of Sm core proteins with small nuclear RNAs (snRNAs) in the biogenesis of small nuclear ribonucleoproteins (snRNPs) means that cells have an obligate requirement for Smn (12–16). After assembly, mature snRNPs are transported into the nucleus where, as part of the spliceosome, they mediate pre-mRNA splicing.

In spite of the identification of the disease gene in 1995 (3), the subsequent discovery of cellular pathways and processes in which Smn is involved, and an abundance of animal models,
RESULTS

Chodl expression is altered in SMA mouse motor neurons

Mouse Chodl encodes three isoforms that share exons 1–5 but differ in their 3′ terminal exon and 3′ UTR usage (Fig. 1A). All three proteins possess an N-terminal signal peptide, a carbohydrate recognition domain, a transmembrane domain and a short cytoplasmic tail (39), which varies between the three proteins (Fig. 1B). Using quantitative RT-PCR (qPCR), we have previously shown that the expression of Chodl-001, but not Chodl-002, is downregulated in SMA mouse whole spinal cord at pre- (postnatal day 1, P1), early- (P7) and late-symptomatic (P13) stages of disease (35). To confirm that these changes relate to motor neurons, we performed isoform-specific in situ hybridization experiments on P7 and P13 lumbar spinal cords of SmnΔ7 (41) and wild-type control littermates. At both time-points in wild-type and SMA mice, Chodl-001 and Chodl-002 displayed robust motor neuron-specific expression (Fig. 1C and D), whereas Chodl-003 was undetectable using two different probes (data not shown). The expression of Chodl-001 in SMA mice is significantly downregulated in the motor neurons at P7 and P13, whereas Chodl-002 levels remain unchanged (Fig. 1E). We therefore focused our studies on Chodl-001.

NSC-34 cells provide a convenient system for Chodl expression studies

Given that Chodl is differentially spliced at pre-symptomatic stages in SMA mouse spinal motor neurons [35] and Fig. 1C and D], we sought to study the effects of reduced Smn levels on Chodl expression in NSC-34 cells, a mouse motor neuronal cell line previously employed in numerous SMA studies (42–45). We confirmed the presence of genomic Chodl DNA in the motor neuronal NSC-34 cell line (Supplementary Material, Fig. S1) but were unable to detect Chodl mRNA via RT-PCR in either undifferentiated or differentiated cells using primers common to all isoforms (Fig. 1F). This was confirmed by qPCR, but not western blotting because the three chondrolectin antibodies tested were insensitive to the mouse protein (data not shown). Therefore, NSC-34 cells serendipitously provide a relevant cellular environment in which to deplete Smn protein by short interfering RNA (siRNA) knockdown and assess the effect of Chodl expression on cellular viability and neurite outgrowth.

Three individual siRNAs targeting different regions of Smn effectively reduced Smn mRNA and protein levels (Supplementary Material, Fig. S2). Smn siRNAs 74016 and 74017 each depleted Smn protein to ≈40 and 20% of control siRNA at 25 and 50 nm, respectively (Fig. 2A and B). SMA is a disease of Smn reduction, not complete absence; hence, it is important that Smn is not depleted below levels [previously shown to be 5–10% (40)] where it would have a non-specific effect in reducing cellular viability by disrupting the constitutive housekeeping function of the protein, which would provide little insight into the relative vulnerability of neuronal cells. Smn protein reduction was also confirmed by immunofluorescence (Fig. 2C and D). The viability of cells was significantly reduced with depleted Smn levels (Fig. 2E), which has been shown previously in NSC-34 cells and a range of other cells including induced pluripotent stem cell-derived motor neurons.
from SMA patients (46–49). A cDNA vector, transiently expressing eGFP-tagged Smn insensitive to siRNA knockdown, completely rescued cell viability, demonstrating that the diminished viability with Smn siRNA administration was not due to non-specific, off-target effects (Supplementary Material, Fig. S3). Smn siRNA 74016 was used in all subsequent experiments and will be known hereafter simply as Smn siRNA.

**Chodl-001 expression exacerbates reduction in cell viability owing to low Smn levels**

To test the effect of increasing chondrolectin levels, we created a cDNA vector to transiently express Chodl-001 with a 3x FLAG tag (Fig. 3A and Supplementary Material, Fig. S4). Chodl-001 expression in cells with normal Smn levels had no effect on...
Chodl expression affects neurite outgrowth in Smn-depleted motor neuronal cells

As zebrafish chondrolectin is involved in axonal growth and development (53), we investigated the effect of Chodl on neurite outgrowth in NSC-34 cells. Smn knockdown in NSC-34 cells has previously been shown to both reduce the percentage of cells that bear neurites and average neurite length (54). Similar phenotypes have been described in induced pluripotent stem cell-derived motor neurons from SMA patients (55), other primary and immortal neuronal cells with reduced Smn levels (56–60), animal models (61,62) and severe SMA patient spinal cords (63).

NSC-34 cells were differentiated using 3% (v/v) serum media, and both the percentage of neurite-bearing cells and average neurite length were calculated at the same time-point that the viability experiments were performed (Fig. 4). With Smn knockdown, the percentage of cells with at least one neurite and the mean neurite length were significantly reduced (Fig. 4A–D). The expression of Chodl-001 had no effect on neurite formation in cells with normal Smn levels, but there was a trend towards viability (Fig. 3B). However, in cells with reduced Smn levels, Chodl-001 expression significantly exacerbated the viability defect caused by Smn depletion. To confirm that the effect of Chodl-001 expression was not simply due to high levels of protein, or a vector artefact, we used the same vector backbone [kindly provided by Esther B. Becker (50)], to express transient receptor potential cation channel, type C3 (Trpc3), a gene not highlighted by exon- or gene-level microarrays in the original SMA mouse study (35). Trpc3 expression had no effect on viability (Supplementary Material, Fig. S5).
decreased neurite length. Doubling the concentration of trans-
ected DNA led to significantly reduced neurite length (Fig. 5A and B and Supplementary Material, Fig. S7).
In cells with diminished Smn, the expression of Chodl-001 at
0.1 μg/well significantly rescued neurite production and length
(Fig. 4C and D), an effect that was negated at twice the concen-
tration (Fig. 5A and B). When measuring neurite lengths, some ex-
tensive processes were counted that may have skewed the results.
Consequently, neurite measurements were also combined for
each experimental treatment and the overall mean and median
values plotted, but the same trends in the data were observed
(data not shown). Moreover, counts were similar between controls
at the different DNA concentrations. We also calculated the per-
centage of cells bearing abnormal neurites (see Materials and
Methods) but saw no major differences (Supplementary Material,
Fig. S8). Together, these observations suggest that chondrolectin
regulates neurite outgrowth dependent upon its expression level
and Smn availability.

**chOdd overexpression rescues motor neuron defects
in Smn-depleted zebrafish**

We have previously shown that dysregulation of zebrafish chodl
expression leads to shorter motor axons and reduced innervation
of myotomes, indicating that chodl is essential for organized
motor axonal outgrowth (53). Similar axonal development and
pathfinding defects are observed with downregulation of Smn
in zebrafish (34,61). Given our in vitro data showing that
Chodl-001 expression in Smn-depleted cells partially corrects
neurite outgrowth defects (Fig. 4), we hypothesized that increas-
ing expression of the single zebrafish isoform of chodl, which
shares the greatest homology with mouse Chodl-001, would
improve the axonal outgrowth defects seen with \textit{Smn} knockdown. Injection of \textit{Smn} morpholinos into \textit{HB9:GFP}\textsuperscript{+} embryos, which express \textit{GFP} in ventrally projecting motor neurons, resulted in abnormal motor axon growth as previously reported (Fig. 6A and B). Co-injection of \textit{chodl} mRNA with \textit{Smn} morpholinos significantly improved the morphology, growth and pathfinding of the motor neurons (Fig. 6C and D and Supplementary Material, Fig. S9). Furthermore, \textit{chodl} expression...
significantly rescued the loss of motor neurons seen in Smn-depleted fish (Fig. 6E). There was no significant difference between the motor axon score of fish injected with 100 and 200 pg chodl mRNA \( (2.07 \pm 0.08 \text{ versus } 1.82 \pm 0.10, P = 0.227, \text{Mann–Whitney } U\text{-test}) \), indicating that a maximal positive effect of chodl is achieved at 100 pg (Supplementary Material, Fig. S9). Together, these experiments confirm that chondrolectin is an \textit{in vivo} modifier of the SMA phenotype.

**DISCUSSION**

Because of the well-established function of Smn in snRNP assembly, it has been proposed that SMA is caused by a general splicing deficiency, or alternatively by mis-regulation of one or a subset of key motor neuron-specific genes (22–25). Nonetheless, defective splicing of a relevant motor neuronal gene at or before disease onset has yet to be identified. Smn depletion results in neurite and axon outgrowth defects in a number of cell lines and animal models (54–62). Furthermore, knockdown of \( Smn \) in human astrogliaoma cells causes defective migration (64), and the spinal motor neurons of patients with severe SMA have been shown to be deficient in migration, axonal outgrowth and ability to increase motor unit territory (63,65). Defects in axonal integrity owing to Smn reduction can be dissociated from loss of snRNP assembly (34).

Using exon-specific microarrays performed on RNA from SMA mouse spinal cords, we previously showed that \textit{chondrolectin} is alternatively spliced at early, pre-symptomatic stages in SMA mouse spinal cord, suggesting that disruption of this or associated pathways may play an important role in the disease (35). Chodl is highly conserved and specifically expressed in

![Figure 5](http://hmg.oxfordjournals.org/)

*Figure 5.* Fine-tuning of Chodl levels is important for neurite outgrowth. (A and B) Doubling the concentration of Chodl-001 abrogates the rescue effect in Smn-depleted cells on the percentage of neurite-bearing cells (A), and neurite length (B) 96 h post-transfection. In cells with normal Smn levels, the increased amount of Chodl-001 exacerbates the associated length deficiency observed at the lower concentration (B). A, \( P = 0.0083 \); B, \( P = 0.0022 \), one-way ANOVA. (C) A representative western blot showing successful Smn depletion and FLAG detection. Chodl was expressed at similar levels between treatments. (D) Densitometric analysis of western blots from three independent experiments reveals that differences in Smn levels do not account for the differential effects of Chodl-001 on neurite outgrowth. \( P = 0.0342 \), Kruskal–Wallis test. *\( P < 0.05 \), **\( P < 0.01 \), Bonferroni’s multiple comparison test. See also Supplementary Material, Figure S7.
developing motor neurons of zebrafish, mice and humans (35–37,53). Defining the function of Chodl in motor neurons may therefore provide important clues about the disease mechanism that links Smn depletion to lower motor neuron loss. After confirming Chodl dysregulation in SMA mouse spinal motor neurons, we used a combination of neuronal cell and zebrafish models to show that chondrolectin has specific effects on neuronal outgrowth and morphology when Smn is depleted.

Chondrolectin is a monomeric transmembrane protein with a C-type lectin-like domain (CTLD) (38,39). These domains typify proteins belonging to the C-type lectin (CLEC) superfamily of proteins (66). The majority of vertebrate CLECs act as recognition molecules within the immune system, because their CTLDs are able to bind to carbohydrates in a calcium-dependent manner. Non-classical CLECs, on the other hand, are able to interact with proteins and other molecules and therefore possess functions outside the immune system. Chondrolectin is not the first CLEC to be implicated in axonal pathfinding—Caenorhabditis elegans CLEC-38 has been shown to function in migrating axons to promote synapse assembly (67). Given that

Figure 6. Overexpression of chodl partially rescues the motor axon defects of Smn-depleted zebrafish. (A–C) Lateral view photomicrographs of HB9:GFP+ zebrafish embryos at 28 h post-fertilization injected with control morpholino (A), Smn (ENSDARG0000018494) morpholino (B) and Smn morpholino with chodl (ENSDARG00000034528) mRNA (C). Control morpholino-injected embryos show hemisegments with wild-type-like ventral motor axons, whereas Smn morpholino-injected fish display numerous bifurcated (arrowhead), truncated (arrow) and missing ventral motor axons (asterisk). chodl mRNA injection with Smn morpholino improves the axonal phenotype caused by Smn knockdown. Scale bars = 50 μm. (D and E) Co-injection of Smn morpholino and chodl mRNA leads to a significant rescue of motor axon outgrowth as assessed by the hemisegment score (see Materials and Methods) (D, *P < 0.001, one-way ANOVA) and the number of motor neuronal HB9:GFP+ cells (E, *P < 0.001, Kruskal–Wallis) as compared with Smn morpholino-injected embryos. *P < 0.05, **P < 0.01, ***P < 0.001, Bonferroni’s/Dunn’s multiple comparison test. See also Supplementary Material, Figure S9.
Chodl is expressed in developing motor nerves and has been implicated in axonal pathfinding, it is likely that its CTLD interacts with extracellular proteins rather than carbohydrates. Using a mouse skeletal muscle cDNA library to identify potential interacting proteins, the cytoplasmic domain of Chodl-001 was shown to bind with the β-subunit of rab geranylgeranyl transferase (Rabggtb) (68). Rabggtb isoprenylates Rab GTPases that are then involved in vesicle trafficking of transmembrane proteins from the Golgi to the cell surface (69,70). Rabggtb is expressed widely during early gestation, but by embryonic day 13.5 (E13.5) is more restricted to the liver and spinal cord (71). Given this expression pattern, Rabggtb is a candidate for interaction with chondrolectin in motor neurons during early development.

In whole mouse embryos, Chodl expression is tightly regulated during early embryonic development, and mRNA levels gradually increase from E7 to E15 but then begin to decline by E17 (39). Chodl is expressed at the base of the developing limb bud and in spinal cord motor neurons at E10.5 and E11.5, a time when the motor axons are reaching and invading the bud region and a number of pathfinding decisions are made (37,72). Given the pattern and timing of expression, it is possible that Chodl is playing an integral role in the development of the nervous system, in particular in motor axon guidance. Corroborating this, our previous work in zebrafish suggests that chodl is vital for the interaction of motor axons with the horizontal myoseptum, an early developmental choice point (53). Moreover, Chodl overexpression increases cellular invasion in a number of different cell lines, concordant with an outgrowth phenotype (73). Nonetheless, increased expression of the single chodl isoform in zebrafish results in abnormal axonal growth similar to morpholino knockdown (53). Similarly, we report that expression of chondrolectin in differentiated NSC-34 cells with normal Smm levels results in reduced neurite outgrowth, the severity of which correlates with DNA concentration (Fig. 4 and 5). The percentage of cells bearing neurites was unaffected by Chodl expression, similar to the observation in zebrafish that chodl overexpression did not cause failure of axon outgrowth. Together, these results suggest that in cells with normal Smm levels, chondrolectin is involved in the outgrowth and elongation of neuronal process, not their initial generation, and that the fine-tuning of Chodl expression is vital for efficient neuronal growth and development, as is the case for many, if not all, axonal guidance genes (74).

The expression of Chodl-001 in Smm-depleted NSC-34 cells reduced viability but improved neurite outgrowth. It is possible that the increased neuronal outgrowth precipitated by Chodl-001 resulted in greater cell death owing to increased pressure to extend neurites in already vulnerable cells. Contrastingly, motor neuron numbers increased when chodl mRNA was co-injected with Smm morpholinos into zebrafish. The natural in vivo environment with its intimate neuronal networks and supporting glial cells may provide the necessary structure and support to cope with the increased demand for nerve growth.

Dysregulation of Chodl, which becomes progressively more prominent during early postnatal development in SMA mice, could lead to the subsequent destabilization and degeneration of neuronal cells early after birth. Consistent with this, no major defects in axonal architecture are seen at embryonic stages in mouse models of SMA (75,76), a time when Chodl dysregulation is just beginning to occur, whereas at P1, pathways involved in postnatal maturation of the spinal cord appear to be disrupted (35). Moreover, CHODL can be found in adult human spinal cord motor neurons (35), suggesting that besides axonal outgrowth and development, chondrolectin may also function in motor neuron maintenance.

In summary, we have identified chondrolectin as an in vivo genetic modifier of the SMA phenotype. Results from Smm-depleted zebrafish suggest the possibility that upregulation of chondrolectin levels could protect motor neurons in SMA. Further work is required to elucidate whether the dysregulation of Chodl seen at pre-symptomatic stages in SMA mouse spinal cord is directly mediated by low Smm levels or is simply an early signature of motor neuron degeneration and loss of neuromuscular integrity.

MATERIALS AND METHODS

General methods

Mouse and zebrafish handling and experiments were performed in accordance with the United Kingdom Home Office Animals (Scientific Procedures) Act (1986). Transgenic Smm<sup>−/−</sup>; Smm<sup>−/−</sup>; Smm<sup>A7</sup> mice (41) were maintained as heterozygous breeding pairs in standard animal facilities in Oxford. Mice were genotyped using tail-extracted DNA and standard PCR procedures. All reagents were obtained from (St. Louis, MO, USA) unless otherwise stated.

In situ hybridization

Target sequences were generated by PCR, cloned into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA, USA), and digoxigenin (DIG)-labelled riboprobes were synthesized from linearized plasmid DNA. Spinal cords were frozen on dry ice in OCT freezing medium (Leica Microsystems, Bensheim, Germany), and 15-µm sections were cut using a cryostat. Probe hybridization, washing and signal detection using an alkaline phosphatase-conjugated anti-DIG antibody were carried out as previously described (77). Sections were developed in parallel for 6 h in all cases to prevent saturation of the expression signal. Data were obtained from three mice of each genotype from four regions of the lumbar spinal cord at least 75 µm apart. Relative expression of Chodl-001 and Chodl-002 was calculated by dividing the total signal intensity in an area of approximately 500 µm<sup>2</sup> (P7) and 600 µm<sup>2</sup> (P13) from ventral horns by the total number of motor neurons. Riboprobe cloning was performed using the following primers: Chodl-001-F 5′ CAA CGA TTC CGC TGC TCT TAC 3′, Chodl-001-R 5′ CGT AGA ATG CCA CTC 3′, Chodl-002-F 5′ TAT AGT GGC CTT CAC ACC G 3′, Chodl-002-R 5′ ACC ATC AGA ATG TTC CGC 3′, Chodl-003-F 5′ CAA GAT CAA ATG TCA AGA TCT G 3′, Chodl-003-R 5′ CTG GCA TGC CTC 3′, and Chodl-004-F 5′ CTG GCA TGC CTC 3′, Chodl-004-R 5′ CTG GCA TGC CTC 3′.

Cell culture and transfection

Mouse NSC-34 (motor neuronal) and C2C12 (myoblastic) cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) with 4.5 g/l D-glucose and pyruvate (Invitrogen) supplemented with 10% (v/v) heat-inactivated foetal bovine serum.
serum (FBS, Invitrogen). The cells were kept at 37°C in a 5% (v/v) CO₂ humidified atmosphere and subcultured every 4–6 days when ∼70–90% confluent. The cells were transfected as previously described (78); NSC-34 cells were grown in 24-well plates in 1 ml DMEM with 10% (v/v) FBS. For experiments performed 72 and 96 h post-transfection, the cells were plated at ≈5.0 × 10⁴ and 2.5 × 10⁴ cells/well, respectively. 24 h post-plating, 600 µl of the media was removed from each well, and the cells treated with 1–1.5 µl of Lipofectamine 2000 Transfection Reagent (Invitrogen) and a final concentration of 25–100 nM siRNA and 0.05–0.5 µg DNA in 100 µl OPTI-MEM Reduced Serum Medium with l-glutamine and HEPES (Invitrogen). 24 h post-transfection, the media was replaced with 1 ml DMEM plus 10% (v/v) FBS. Silencer Select pre-designed siRNAs targeting Smn were ordered from Applied Biosystems, Inc. Foster City, CA, USA: s74016 sense 5’ UUU ACC CAG CUA CUA tt 3’ and reverse 5’ GCA TCG CCT CAC TCT TGC G 3’. Cycling conditions were as follows: 95°C for 30 s, 18 × [95°C for 30 s, 55°C for 1 min, 70°C for 9 min], 70°C for 2 min. The mutated construct was gel-purified and verified by sequencing using CMV_F.

Cloning of 3×FLAG-Chodl-001 cDNA construct
One microgram of RNA extracted from the tibialis anterior muscle of a young mouse was reverse-transcribed in a 15-µl reaction mixture using Taq-Man Reverse Transcription Reagents (Applied Biosystems, Inc.). Chodl cDNA was then amplified in a 25 µl PCR with KOD Hot Start DNA Polymerase (Novagen, Inc., Madison, WI, USA) using sequence-specific primers with added 5’ HindIII and XbaI restriction sites: Chodl-001 forward 5’ GCA AGC TTA TGA TCC CCT CAC TCT TGC 3’ and Chodl-001 reverse 5’ GGA AGG AAA GTG GCA TGG AGG TAT AAT CTA GAT ATA 3’. The following PCR cycling conditions were used: 95°C for 2 min, 40 × [95°C for 20 s, 62°C for 10 s, 70°C for 20 s], 70°C for 2 min. PCR products were digested for 2 h at 37°C with HindIII and XbaI (New England Biolabs, Inc., Beverly, MA, USA) and were column-purified using a PCR purification kit (QIAGEN, Valencia, CA, USA). The p3×FLAG-CMV-7.1 expression vector (E7533) was cut using the same restriction endonucleases, which were then heat-inactivated for 20 min at 65°C. To prevent recircularization, the digested vector was treated with 1 µl calf intestinal phosphatase (New England Biolabs, Inc.) for 1.5 h at 37°C and gel-purified. In a 20-µl reaction mixture, 50 ng of vector and an equimolar amount of insert were ligated using T4 ligase (New England Biolabs, Inc.) for 1.5 h at room temperature. Plasmid maxi-preps (QIAGEN) were ligated using T4 ligase (New England Biolabs, Inc.) for 1.5 h at 37°C and gel-purified. In a 20-µl reaction mixture using Taq-Man Reverse Transcription Reagents (Applied Biosystems, Inc.), Chodl cDNA was then amplified in a 25 µl PCR with KOD Hot Start DNA Polymerase (Novagen, Inc., Madison, WI, USA) using sequence-specific primers with added 5’ HindIII and XbaI restriction sites: Chodl-001 forward 5’ GCA AGC TTA TGA TCC CCT CAC TCT TGC 3’ and Chodl-001 reverse 5’ GGA AGG AAA GTG GCA TGG AGG TAT AAT CTA GAT ATA 3’. The following PCR cycling conditions were used: 95°C for 2 min, 40 × [95°C for 20 s, 62°C for 10 s, 70°C for 20 s], 70°C for 2 min. PCR products were digested for 2 h at 37°C with HindIII and XbaI (New England Biolabs, Inc., Beverly, MA, USA) and were column-purified using a PCR purification kit (QIAGEN, Valencia, CA, USA). The p3×FLAG-CMV-7.1 expression vector (E7533) was cut using the same restriction endonucleases, which were then heat-inactivated for 20 min at 65°C. To prevent recircularization, the digested vector was treated with 1 µl calf intestinal phosphatase (New England Biolabs, Inc.) for 1.5 h at 37°C and gel-purified. In a 20-µl reaction mixture, 50 ng of vector and an equimolar amount of insert were ligated using T4 ligase (New England Biolabs, Inc.) for 15 min at room temperature. Plasmid maxi-preps (QIAGEN) were prepared according to the manufacturer’s instructions, and constructs were gel-purified and verified by DNA sequencing using CMV_F 5’ GAG CTC GTT TAG TGA ACC GTC 3’ and mChodl_Seq_F 5’ ACC AGT GGT GTG CTG AAG GC 3’.

pEGFP-N1-Smn site-directed mutagenesis
Site-directed mutagenesis of a pEGFP-N1-Smn vector (Clontech, Palo Alto, CA, USA) was performed using a QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA). Primers were designed according to the manufacturer’s instructions and had high-performance liquid chromatography-purified. Over two rounds of mutagenesis, three base-pair changes were incorporated into the target sequence of Smn siRNA 74016 using the following primers (with mutations underlined and bold, and the siRNA target sequence in lowercase): Smn_mut_1a 5’ CTT TCC Ccg aca tgg gaa gtt gct aat AG TAC 3’, Smn_mut_1b 5’ GTA CTA tta gca act tca cat gtc ggg GAA AG 3’, Smn_mut_2a 5’ cga cat gtg aag tgt cca aTA Gta CAG AAC AGA ACA CTC AGG 3’ and Smn_mut_2b 5’ CCT GAG TGT TCT GTT CTG TAC Tat tgt cca ctt cac atg teg 3’. Cycling conditions were as follows: 95°C for 30 s, 18 × [95°C for 30 s, 55°C for 1 min, 70°C for 9 min], 70°C for 2 min. The mutated construct was gel-purified and verified by sequencing using CMV_F.

DNA extraction and standard PCR
The cells were collected in 1.5-ml eppendorf tubes using 1× phosphate-buffered saline (PBS, 137 mM NaCl, 10 mM Na₂HPO₄, 2.7 mM KCl, 2 mM KH₂PO₄), spun at 4000 × g for 2 min, resuspended in DNA extraction buffer [200 µl NaCl, 100 mM Tris–HCl (pH 7.5), 5 mM EDTA (pH 8.0), 0.2% (w/v) sodium dodecyl sulphate (SDS)] with 3 µl freshly added 20 µg/ml proteinase K (Roche Diagnostics, Indianapolis, IN, USA) and left at 55°C overnight. To extract DNA from mouse tissue, no centrifugation step was required. The following day, the samples were spun at 16,000 × g for 10 min, the supernatants collected and an equal volume of isopropanol added. DNA was pelleted at 16,000 × g for 10 min, air-dried for 5–10 min and then resuspended in 50 µl H₂O. Per 20 µl PCR with Taq DNA polymerase, 15–25 ng genomic DNA was used. Chodl primer sequences for a product of 701 bp were as follows: forward 5’ CCA GAC ACG CCC ACG GTG GCA CCT GAC ACG CCC ACG GTG TGC 3’ and reverse 5’ GGC GTT CAT GCC ACA GCA GC 3’. Trpc3 primer sequences for a product of 859 bp were as follows: forward 5’ GTA GCA ACT AGC CCC TT 3’ and reverse 5’ TGG AAC TGC GAA CAT CAC CA 3’. Cycling conditions were as follows: 95°C for 3 min, 35 × [95°C for 30 s, 64°C for 30 s, 72°C for 1 min], 72°C for 2 min. PCR products were separated on a 1.5% (w/v) agarose gel.

RNA extraction and reverse transcription PCR (RT-PCR)
RNA was extracted from cell lines and P5 mouse tissues using an RNeasy Mini Kit (QIAGEN) following the manufacturer’s instructions. In each reverse transcription reaction, 1–1.5 µg RNA/15-µl reaction mixture was added. The cDNA was diluted 1/10 and 1 µl was used per 20 µl PCR with Taq DNA polymerase. Chodl primer sequences for a product of 723 bp were as follows: forward 5’ GCA TCG CCT CAC TCT TGC TGG 3’ and reverse 5’ GCA ACA TCT GGA AAG AGG 3’. Gapdh primer sequences for a product of 486 bp were as follows: forward 5’ GTA TGT CGT GAA GTC TAC TGG 3’ and reverse 5’ TAC TTT GCA GGT TTC TCC TCC AGG 3’. Trpc3 primer sequences for a product of 620 bp were as follows: forward 5’ AGA TCT CTC TTT GGA GAA C 3’ and reverse 5’ TGT TGG CTG ATT GAG AAT G 3’. Cycling conditions were as follows: 95°C for 3 min, 34 × [95°C for 30 s, 61/64/58°C for 30 s, 72°C for 1 min], 72°C for 2 min. RT-PCR products were separated on a 1.5% (w/v) agarose gel.
Quantitative RT-PCR
To create cDNA, 100 ng RNA/15-μl reaction mixture was used. The cDNA was diluted 1/10 and 1 μl used per 20 μl qPCR using SYBR Green (Applied Biosystems, Inc.) and a StepOnePlus real-time PCR machine (Applied Biosystems, Inc.). Primer sequences were as follows: Gapdh forward 5′ TGT GTC GCT CGT GGA TCT GA 3′ and reverse 5′ CTT GCT TCA CCT TCT TGA 3′; Smn forward 5′ CGA GAA GAA AAC CTG CCA AGA A 3′ and reverse 5′ CAC TTC GAC CAC ACT TTC CAC TGT 3′. Cycling conditions were as follows: 95°C for 10 min, 40 × [95°C for 15 s, 60°C for 1 min], 95°C for 15 s. Three technical replicates per reaction were performed, and primers were used at 200 nM. Primer efficiencies were estimated by performing qPCR on serial dilutions of at least two independent cDNA samples, plotting Ct values on the Y-axis against log-transformed cDNA inputs, and using the slope of the line of regression in the following equation: (10^{−1/slope} − 1) × 100. Relative gene expression was calculated using the comparative Ct (ΔΔCt) method and Gapdh as the reference gene: ΔCt = CtSmn − CtGapdh, and ΔΔCt = ΔCtexperimental sample − ΔCtcontrol sample, with relative expression given using the formula 2^{−ΔΔCt}. Ct values were adjusted for primer efficiency using the following formula: Ct value × [log(2 × primer efficiency/100)/log(2)].

Protein extraction and western blotting
The cells were re-suspended in PBS, spun at 4000 × g for 2 min, re-suspended in 50 μl radioimmunoprecipitation assay lysis buffer [150 mM NaCl, 50 mM Tris–HCl (pH 7.5), 1% (v/v) sodium deoxycholate, 1% (v/v) Triton X-100, 0.1% (w/v) SDS] with freshly added 1.5% (v/v) protease inhibitor cocktail and left on ice for 30 min. The cells were spun at 3800 × g for 10 min and the supernatant transferred to fresh tubes, which were then stored at −80°C. Protein concentration was quantified using a Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA), as per the manufacturer’s instructions. For western blotting, approximately equal amounts of protein (10–20 μg) from each sample were boiled for 5 min in Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) with 5% (v/v) β-mercaptoethanol and separated by 10% (w/v) SDS polyacrylamide gel electrophoresis. Resolved gels were transferred to 0.2 μm nitrocellulose membranes (Millipore Corporation, Bedford, MA, USA), which were then blocked for 1 h in 5% (w/v) milk powder in PBS and incubated overnight with primary antibody in 5% (w/v) milk in PBS–Triton (PBS-T, 0.1% (v/v) Triton X-100 in PBS) at 4°C on a rocker. Rabbit polyclonal anti-β-tubulin was purchased from Abcam (ab15568, Cambridge, UK) and used at 1/5000, mouse monoclonal antiFLAG from Sigma (F1804) at 1/1000, and mouse monoclonal anti-Smn from BD Biosciences (610646, Franklin Lakes, NJ, USA) at 1/5000. The following day, membranes were washed three times for 10 min in PBS-T, probed with horseradish peroxidase-conjugated secondary antibodies (Amersham Corporation, Arlington Heights, IL, USA) at 1/5000 in PBS-T with 5% (w/v) milk for 2 h and washed three times for 10 min in PBS-T. Signal was detected using enhanced chemiluminescence reagents (GE Healthcare) according to the manufacturer’s instructions. For probing with a second primary antibody, membranes were stripped at 55°C for 30 min in stripping buffer [100 mM β-mercaptoethanol, 62.5 mM Tris–HCl (pH 6.8), 2% (w/v) SDS] and washed three times for 10 min with PBS-T, and the probing process repeated starting with blocking for 1 h in 5% (w/v) milk in PBS. Densitometric quantification in the linear range was performed using the Gels tool of ImageJ (http://rsweb.nih.gov/ij/, accessed 30 September 2013). The loading control (β-tubulin for all Westerns) band density of each sample was normalized to the same single sample to produce a normalization factor. The density of each Smn band was then multiplied by the normalization factor for each individual sample. These values were then used to calculate the percentage Smn levels relative to an experimental control condition (the first bar in each graph).

Immunocytochemistry
The cells were grown in wells of a 24-well plate on 13-mm circular coverslips coated for 10 min with 10 μg/ml poly-d-lysine (BD Biosciences). The coverslips were removed with forceps, rinsed in a few drops of PBS and fixed with freshly prepared 4% (w/v) paraformaldehyde (PFA, Electron Microscopy Sciences, Hatfield, PA, USA) in PBS for 15 min. The PFA was aspirated from the cells, which were then bathed for 10 min in PBS, permeabilized with a few drops of 0.3% (v/v) Triton X-100 in PBS for 20 min, washed three times for 5 min in PBS and blocked for 1 h in 5% (w/v) milk in PBS. Primary antibodies in 150–200 μl 15% (w/v) milk in PBS were then added to the cells and left overnight at 4°C. Anti-β-tubulin was used at 1/500, and anti-Smn at 1/200. The following day, the cells were washed three times with PBS for 10 min and incubated for 2 h at room temperature with secondary antibodies (Alexa Fluor 488/594 goat anti-Rabbit and Alexa Fluor 488/594 goat anti-mouse, Invitrogen) at 1/1000 in PBS. The cells were washed three times for 10 min with PBS, mounted using Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA) and imaged using a Zeiss LSM 510 META (Oberkochen, Germany) laser scanning microscope.

MTT viability assay
The enzymatic cleavage of thiazolyl blue tetrazolium bromide (MTT) can be used as a proxy for cell viability (51,52); soluble, yellow MTT is converted to insoluble, purple formazan crystals by dehydrogenase enzymes in functioning mitochondria. Media was aspirated from the cells, which were then incubated for 30–50 min at 37°C with 250 μl 500 μg/ml MTT in PBS. The assay was terminated and the formazan solubilized using 600 μl dimethylsulphoxide. To aid solubilization, the plate was put in a 37°C shaker for 5–15 min. To stop residue formation in the wells affecting the absorbance reading, 500 μl of this solution was then transferred to a new 24-well plate. At least three wells without cells were processed in parallel. Absorbance of each well was measured at a wavelength of 570 nm using a FLUOstar OPTIMA plate reader (BMG Labtech, Ortenberg, Germany). The absorbance of each well was recorded at least three times and a mean average taken. To calculate relative viability, the mean absorbance of the wells lacking cells was subtracted from the mean recording for each individual well. This individual value was then used to calculate
viability relative to the mean average across all the wells of a control treatment (the first bar in each graph).

Trypan blue exclusion assay

The cells and media of each well were collected into 1.5-ml eppendorf tubes. The cells were pelleted at 4000 x g for 2 min, the supernatant carefully removed and the cells then resuspended in 100 μl 0.4% (w/v) trypan blue/serum-free DMEM (1:1). To a haemocytometer, 10 μl of this cell suspension was then applied and viewed using an Olympus CK2 inverted microscope (Tokyo, Japan) with 10 x objective. The percentage of the viable cells was calculated by dividing the number of live cells (as assessed by exclusion of the trypan blue dye) by the total number of cells and multiplying by 100.

NSC-34 neurite measurements

Undifferentiated NSC-34 cells are round and lack neuronal processes; therefore, neurite outgrowth was induced by reducing FBS concentration of the supporting media from 10 to 3% (v/v), a concentration shown to reduce serum withdrawal-associated cell death (79). After transfection, the cells were allowed to grow for 96 h in differentiation media. β-tubulin-stained cells were examined using a Zeiss Axioplan 2 microscope with mounted AxioCam and a 20 x objective lens. At least 15 images were taken of the cells from each condition per individual experimental replicate, and an average of 121.4 cells were counted to assess the percentage of cells bearing at least one neurite, and 59.4 neurites measured to calculate mean length. Image fields were selected at random, and experiments were blinded. The cells in direct contact with five or more other cells and neurites of <10 μm were excluded from the analyses. Neurite measurements were calculated using ImageJ by manually plotting points along the length of a cell’s longest neurite. Abnormal neurites were scored as those with axonal swellings, that completely reverted back on themselves or that diverged off at a distinct angle of >30° at least 10 μm from the base of the projection (80).

Antisense Smn morpholino and synthetic chodl mRNA injections in zebrafish embryos

Zebrafish embryos were maintained using standard protocols at 28.5°C and staged by hours post-fertilization (hpf) (81). Tg(tmn1:GFP)ml2 abbreviated as HB9:GFP+ zebrafish (82), which express GFP in ventrally projecting motor neurons, were used for morpholino and mRNA injections. An antisense morpholino was generated against the 5’ start sequence of the zebrafish Smn gene (Gene Tools, Philomath, OR, USA); 5’ CGA CAT CTT CTG ACC CAT TGG C 3’ (61). Controls of specificity for the antisense Smn morpholino have been previously reported (61). One-to four-cell HB9:GFP+ embryos were injected with a 0.5 mM morpholino solution in water or in water containing 100 or 200 ng/μl synthetic zebrafish mRNA, equating to ~100 and 200 pg/embryo, respectively. Rhodamine-conjugated dextran (Invitrogen) was added to morpholino solutions as a positive marker for successful injections. The synthetic zebrafish chodl and transposase mRNA was produced using an mMESSAGE mMACHINE kit (Life Technologies, Carlsbad, CA, USA) as previously described (53,83). A standard control (Gene Tools) morpholino (5’ CCT CTT ACC TCA GTT ACA ATT TAT A 3’) was used in the same concentration for control injections.

After injections, the phenotype of the embryos was analysed at 28 hpf by using a scoring system similar to those previously used by others (34,61,84). For the visualization of GFP transgenic fish, live HB9:GFP+ embryos were anesthetized with MS-222, fixed for 45 min with 4% (w/v) PFA in PBS and mounted on glass coverslips using 70% (v/v) glycerol in PBS. Scoring of the hemisegment phenotypes was done by fluorescence microscopy using a Zeiss AxioPlan 2 microscope and a 20 x objective. For scoring, 16–20 hemisegments were assessed per embryo (8–10 per side) according to the following classification: (0) for a hemisegment without a ventral motor axon (not distinguishing between missing motor neurons or missing axons); (1) for a hemisegment with a ventral motor axon that extended maximally to the horizontal myoseptum; (2) for a hemisegment with a ventral motor axon that had grown beyond the horizontal myoseptum and bifurcated, had abnormal additional branches or failed to stay on the mid-segmental pathway; (3) for a hemisegment with a wild-type-like ventral motor axon. A wild-type ventral motor axon is defined as one that has grown beyond the horizontal myoseptum and has only small terminal branches. The mean score was then calculated for each embryo. Embryos from four independent experiments were scored. The survival of the embryos was always >75% in the three groups. For quantification of HB9:GFP+ neurons, the trunk region of the embryos was scanned with a 20 x objective by confocal microscopy (LSM710, Zeiss). All fluorescent neurons in trunk segments 6 and 7 were counted in three dimensions using confocal image stacks of the spinal cord. Two independent experiments were counted.

Statistical analysis

When normally distributed, data sets were statistically analysed using a one-way analysis of variance (ANOVA) with Bonferroni’s multiple comparison test. If the data did not pass normality testing, the non-parametric Kruskal–Wallis test with Dunn’s multiple comparison test was used. For regression analysis, linear regression was performed and R2 values calculated. Pearson’s product–moment correlation coefficient (R) was calculated to assess correlation. GraphPad Prism 5 software was used for all statistical analyses. Means ± standard errors are plotted for all graphs unless otherwise stated.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

AUTHOR CONTRIBUTIONS


All authors have read and approved submission of this work.
ACKNOWLEDGEMENTS

The authors thank Neza Alfazema, Andrew R. Bassett, Esther B. Becker, Dirk Bäumer, M. Zameel Cader, Carmen H. Coxon, Stuart J. Grice, Zoltán Molnár, Tamara M. Sirey and Gregory A. Weir for experimental advice, fruitful discussions and providing reagents.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by grants from the Spinal Muscular Atrophy Trust and the Motor Neurone Disease Association (K.T. Laboratory). Additional funding was supplied by the Medical Research Council (J.N.S., P.L.O., A.B., K.E.D.), and the Fundación Barrié (A.B.-I.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

REFERENCES


