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Alpha-Synuclein Loss in Spinal Muscular Atrophy

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Abstract

Spinal muscular atrophy, the most prevalent hereditary motor neuron disease, is caused by mutations in the survival motor neuron (*SMN*) 1 gene. A significant reduction in the encoded SMN protein leads to the degeneration of motor neurons. However, the molecular events leading to this process are not well understood. The present study uses a previously developed neuronal cell culture model of spinal muscular atrophy for a multiplex transcriptome analysis. Furthermore, gene expression analysis was performed on in vitro cell cultures, as well as tissue samples of spinal muscular atrophy patients and transgenic mice. RNA and subsequent Western blot protein analyses suggest that low SMN levels are associated with significantly lower alpha-synuclein expression. Examination of two genes related to vesicular transport showed a similar though less dramatic decrease in expression. The 140-amino acid protein alpha-synuclein, dominant mutations of which have previously been associated with an autosomal dominant form of Parkinson's disease, is strongly expressed in select neurons of the brain. Although not well understood, the physiologic functions of alpha-synuclein have been linked to synaptic vesicular neurotransmitter release and neuroprotection, suggesting a possible contribution to *Smn*-deficient motor neuron pathology. Furthermore, alpha-synuclein may be a genetic modifier or biomarker of spinal muscular atrophy.

Keywords

Spinal muscular atrophy; Alpha-synuclein; Motor neuron disease; Survival motor neuron

Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive condition characterized by selective degeneration and loss of lower motor neurons, leading to proximal weakness and skeletal muscle atrophy (Murayama et al. 1991). SMA is the most prevalent hereditary motor neuron disease, affecting as many as four to ten per 100,000 live births (Burd et al. 1991), with one in 40–75 of all people being carriers of the genetic mutation. There is no specific treatment or cure available.

SMA is caused by homozygous deletion of exon 7 or, less frequently, other mutations in the telomeric copy of the survival motor neuron (*Smn*) 1 gene on chromosome 5q13 (Brzustowicz et al. 1990). The clinical severity of SMA has been associated with the copy number and/or level of expression of centromeric *SMN2* (Wirth 2000), which differs from *SMN1* by at least a single nucleotide change which results in altered splicing of the transcript (Lorson et al. 1999; Monani et al. 1999). Immunoblot analysis of fibroblast samples suggested SMN protein from type I SMA patients is decreased to between 8% and 21% of controls, while in the intermediate form, type II, SMN protein levels were between 31% and 100% (Lefebvre et al. 1997). Furthermore, in a type I SMA patient, SMN protein levels were 30% of that seen in control fibroblasts (Nguyen et al. 2008). However, there are no human data available for SMN protein levels in the anterior horn cells of SMA patients. Transgenic mice with a severe (type I) SMA phenotype expressed 10–20-fold lower full-length SMN protein in the spinal cord than their control littermates (Monani et al. 2000). However, other genetic modifiers are suspected to play a role in determining disease severity and progression (Oprea et al. 2008).

The SMN protein is present in all cell types, and its complete absence causes early embryonic death by massive apoptosis (Schränk et al. 1997). Most cells tolerate a low level of SMN, except anterior horn cells (reviewed by (Burghes and Beattie 2009)). SMN is present in the nucleus and cytoplasm, in association with ribonucleo-protein complexes that play a role in RNA processing (Fischer et al. 1997). SMN is also localized in axons, including axonal growth cones (Fan and Simard 2002). Decreasing SMN during early development causes aberrant guidance of motor axons in Zebrafish (McWhorter et al. 2003). SMN has been shown to play a role in axonal transport of β -actin mRNA (Eggert et al. 2006). However, it is unclear how SMN affects functional integrity in anterior horn cells and their axons (Gubitza et al. 2004), although our previous work showed that SMN overexpression protects neuronal cell from induced apoptosis (Parker et al. 2008). Recently, more emphasis was directed to the pathology of neuromuscular junctions (NMJ) in animal models of SMA. Simplified NMJ with presynaptic defects were found in a transgenic mouse model of SMA (Kariya et al. 2008; McGovern et al. 2008; Kong et al. 2009). Furthermore, impaired synaptic vesicular release and neurotransmission was suggested, along with an observed decrease of a synaptic vesicle protein (SV2) in transgenic SMA models (Chan et al. 2003; Jablonka et al. 2007; Boon et al. 2009). It is still unclear how defects in the ubiquitously expressed SMN result in selective lower motor neuron degeneration leaving other neuronal cells and tissues ostensibly unaffected.

Mouse NSC-34, P19, and rat pheochromocytoma cells (PC12) are currently the most frequently used in vitro models of developing motor neurons in SMA research (Trülsch et al. 2004, 2007; Bowerman et al. 2007; Liu et al. 2010; Wen et al. 2010) because of the difficulty in maintaining and manipulating primary neurons in vitro (Cashman et al. 1992). NSC-34 cells respond in a comparable manner to developing motor neurons to voltage-gated ion channel modulation (Matsumoto et al. 1995) and demonstrate similar cytoskeletal organization and axonal transport (Cashman et al. 1992; Matsumoto et al. 1995). We have performed an initial screening experiment using a gene expression array associated with

“neurodegeneration.” The results indicated a previously unreported reduction in expression levels of alpha-synuclein (SNCA) in NSC-34 cells bearing a stable low level of SMN. Further analyses of SMA patient fibroblast and spinal tissue samples presented here suggest that low SMN levels are associated with significantly lower mRNA and protein levels of SNCA. The neuronal function of normal SNCA has been associated with vesicular transport (Utton et al. 2005; Roy et al. 2008) and synaptic transmission in neurons (Utton et al. 2002), and hence may contribute to the axonal and NMJ pathology described in SMA.

Materials and Methods

Generation of pLKO.1-puro-Smn-shRNA Expression Plasmids

Murine Smn mRNA sequence (accession: NM_011420) was used to select the target regions for shRNA gene silencing. Five individual clones of pLKO.1-puro-Smn-shRNA were selected and generated by Sigma. The pLKO.1-puro vector is a lentivirus plasmid vector containing U6 promoter and bacterial (ampicillin) and mammalian (puromycin) antibiotic resistance genes for selection of insert in either bacterial or mammalian cell lines. The five oligonucleotide sequences used to construct shRNA were (1) CCGGGCTCTAAAGAACGGTGACATTCTCGAGAATGTCACCGTTCTTTAGAGCTT TTTG, (2) CCGGCCCTTGAAACAGTGGAAAGTTCTCGAGAACTTTCCACTGTTTCAAGGGTT TTTG, (3) CCGGCCATTGACTTTTAAGAGAGAACTCGAGTTTCTCTCTTAAAGTCAATGGTTT TTTG, (4) CCGGCGACCTGTGAAGTAGCTAATACTCGAGTATTAGCTACTTCACAGGTCGTT TTTG, and (5) CCGGCTCTTGGTACATGAGTGGCTACTCGAGTAGCCACTCATGTACCAAGAGTT TTTG.

NSC-34 Cell Culture and Transfection

NSC-34 cells (Cashman et al. 1992) were grown in Dulbecco's modified Eagle medium (Gibco) supplemented with 10% fetal bovine serum with 100 units/ml penicillin and 100 µg/ml streptomycin at 37°C. Vector transfection of the individual clones of pLKO.1-puro-Smn-shRNA was carried out by Lipofectamine 2000 (Invitrogen). The day before transfection, 2–4×10⁵ cells per well were seeded on 6-well plates. The plasmid concentration for each well was 4.0 µg. Clones were selected and grown as described in Acsadi et al. (2009). Transient transfection was also performed in separate cultures using “stealth” siRNA (Invitrogen) under conditions previously optimized with a final siRNA concentration of 100 nM (Parker et al. 2008). Briefly, siRNAs were synthesized that target bases 456–483 (Invitrogen; Carlsbad, CA, USA): sense *Smn* siRNA was 5'-GACCUGUGAAGUAGCUAAUAGUACA-3', antisense *Smn* siRNA was 5'-UGUACUAUUAGCUACUUCACAGGUC-3', sense control siRNA was 5'-GACAGUGAUGAAUCGGAUUAUUCACA-3', and antisense control siRNA was 5'-UGUGAAUAUCCGAUUAUCACUGUC-3'. The *Smn* specific siRNA sequence was BLASTed (NCBI database), and a control siRNA sequence was used to support the contention that solely the *Smn* gene was targeted. Additional siRNA sequences previously targeted to a different region were performed that yielded a similar Smn knockdown efficiency. Cells were assayed after 72 h in culture.

Human SMA Fibroblast and Spinal Cord Samples

Human untransformed fibroblasts of an SMA type 1 patient, an SMA carrier (SMA patient's mother), and a healthy control were obtained from Coriell Institute tissue repository (Camden, NJ, USA). Spinal cord RNA samples were also provided by the University of

Utah tissue repository (Clinical and Genetic Analysis of Spinal Muscular Atrophy, IRB no. 00008751). RNA samples were examined for quality and used for quantitative reverse transcription-polymerase chain reaction (RT-PCR) to compare SMN and SNCA levels in controls: 4 MO X-linked SMA patient, 5 MO spinal muscular atrophy respiratory distress disease (SMARD) patient, and 15 MO who succumbed to pesticide exposure, all with normal SMN1 dosage, and three SMA type 1 patients: 5 MO, 15 MO, and 47 MO.

PCR Array

StellARray™ Gene Expression Plate “Neurodegeneration” (NE0100-MM96) was used for the PCR array analysis (Bar Harbor BioTechnology, Trenton, ME, USA). One microgram of total cellular RNA was extracted from the NSC-34 C-2 cell clone with low SMN and compared to a control NSC-34 clone. Total RNA was reverse transcribed in a final reaction mix of 20 µl using Taq-Man RT reagents (Applied Biosystems) according to the manufacturer's instructions. cDNA was diluted to a concentration of 100 µg/µl. For one 96-well plate of the PCR array, 1,050 µl of 2× SYBR Green, 900 µl of RNase free water, and 210 µl of diluted cDNA were prepared, and aliquots of 20 µl was added to each well. Universal cycling conditions (10 min at 95°C, 15 s at 95°C, 1 min 60°C for 40 cycles) were carried out for PCR. Data normalization and analysis were carried out according to global pattern recognition analysis tool.

Quantitative RT-PCR

Total RNA from transfected cells was extracted using RNeasy Mini Kit (QIAGEN) following the manufacture's protocol. Total RNA (1 µg) was used to synthesize cDNA 20 µl using Taq-Man RT reagents (Applied Biosystems) according to the manufacturer's instructions. The reactions were incubated at 25°C for 10 min, 48°C for 30 min, 95°C for 5 min, and soaked at 4°C. Quantitative real-time PCR (qRT-PCR) for target genes and internal control GAPDH were carried out using MJ DNA Engine Opticon System (MJ Research Inc.). Twenty-five-microliter PCR reactions were performed with SYBR Green I dye (Applied Biosystems), 300 nM primers, and 300 ng cDNA. The primer sequences were mouse *Smn* forward 5'-TTCCCCGACCTGTGAAGTAG-3' and reverse 5'-GGCTTTCCTGGTCCCTAATCC-3'; mouse α -synuclein forward 5'-AGTTGTGGCTGCTGCTGAGAAA-3' and reverse 5'-AGCCACTGTTGCTACTCCATGA-3'; mouse *Sv2a* forward 5'-GTGAACAGCACGTTTCTGCACAAT-3' and reverse 5'-CAACACACTGGAACCAGCAAGCAT-3'; mouse *Syn2* forward 5'-ACCTTCATCGACGCCAAGTATGA-3' and reverse 5'-CAGGCCACCAACATTTTCAGAGCA-3'; mouse *GAPDH* forward 5'-GTGTTCTTACCCCCAATGTG3'- and reverse 5'-AGGAGACAACCTGGTCCTCA3'; human *SMN* forward 5'-GCTGATGCTTTGGGAAGTATGTTA-3' and reverse 5'-AATGTGAGCACCTTCCTTCTTTTT-3'; human α -synuclein forward 5'-ACAAGTGCTCAGTTCCAATGTGCC-3' and reverse 5'-GTGAAAGGGAAGCACCGAAATGCT-3'; human *SV2A* forward 5'-CGCCTTTCCTTCTGTGTTTGCCAT-3' and reverse 5'-TGTGGGTTACTGAGAACTCGCT-3'; human *SYN2* forward 5'-ACTTCCGCCACCTGATCATTGGTA-3' and reverse 5'-ATAGATAGCGACCAGCTGGGCAAA-3'; human *GAPDH* forward 5'-TGGACAGTCAGCCGCATCTTCTTT-3' and reverse 5'-ACCAAATCCGTTGACTCCGACCTT-3'.

The thermal cycling conditions are 50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 15 s, and 60°C for 1 min. Experiments were performed in triplicate for each data point. Relative gene expression was calculated using the comparative Ct method. Quantitative

normalization of cDNA in each sample was performed using expression of GAPDH as an internal control.

SNCA Western Blot

Protein from cells was extracted with 2% sodium dodecyl sulfate and 20 µg loaded. Sixteen percent Tris-glycine gels were used for protein separation then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Corporation). Membranes were incubated with rabbit anti- α -synuclein IgG antibody (1:500, Signalway antibody) and polyclonal rabbit anti- β -actin IgG (1:8,000, Sigma) at 4°C overnight. A horseradish peroxidase (HRP) goat anti-mouse IgG was the secondary antibody (1:5,000, Pierce, Rockford, IL, USA) incubated at room temperature for 1 h, visualized by chemiluminescence substrate (Pierce), and images obtained by X-ray exposure. The intensity of western blot bands was measured by Kodak digital science 1D Image Software, and synuclein/ β -actin density ratios were compared.

Results

Development of NSC-34 Cells with Low SMN Levels

Real-time RT-PCR was used to assess *Smn* gene expression in *Smn* shRNA transfected NSC-34 cell clones at day 5 (Fig. 1a). For example, clones C-14, C-11, and C-10 showed a decrease in *Smn* gene expression to 74.4%, 52.7%, and 36.4%, respectively, relative to control transfected NSC-34 cells. Semi-quantitative western blot analysis of SMN protein levels in clones C-14, C-11, and C-10 at day 5 (Fig. 1b) shows, respectively, 23.3%, 49.8%, and 87% decrease in SMN protein levels relative to control transfected NSC-34 cells.

Clone C-2 was selected for further analysis on the criteria that it had a decrease in SMN levels consistent with those observed in SMA mouse models and human SMA fibroblast samples. Not all clones show consistency with passaging, hence the need for validation of downregulation throughout an experiment. qRT-PCR for SMN at passages 5 and 7 revealed 70% and 74% decrease in SMN mRNA levels relative to NSC control corrected for GAPDH (not shown). Western blot for *Smn* protein at passages 5 and 7 relative to NSC control corrected for β -actin is shown (Fig. 1c, 47% of control ratio of SMN/ β -actin 1.56 and 1.50 at passages 5 and 7, respectively). Blots were run on the same gel, but the image has been cropped for clarity. Clone C-6 was selected for further analysis as its levels of SMN expression were lowered compared to control but not as dramatically as clone C-2 and not as low as has been required to observe SMA pathology in patients and mouse models. Semi-quantitative analysis of the western blot showed a ratio of 1.897 and 1.909 for SMN/ β -actin at the passage used compared to two passages earlier, indicating no increase in SMN protein level in the C-6 clone (figure not shown).

qRT-PCR Array for Known Genes Participating in “Neurodegeneration”

Total cellular RNA was extracted from the C-2 cell clone with low SMN and compared to a control clone using a “neurodegeneration” qPCR plate array (StellARay™ Gene Expression Plate; NE0100-MM96; Bar Harbor BioTechnology, Trenton, ME, USA). The genes that had more than twofold change on the plate array are listed in Table 1. *Snca* expression was the most significantly regulated (>30-fold reduction, $p < 0.0005$). The only other gene indicated on the neurodegeneration plate with a link to muscular atrophy was *Hap1*, which has been associated with Huntington's disease and spinal and bulbar muscular atrophy (Takeshita et al. 2006).

qRT-PCR of SMN and SNCA in Human SMA Fibroblasts and NSC-34 Clones

In fibroblasts sampled from SMA patients, the *SMN1* mRNA level showed a 45% decrease from control to type III patients (Fig. 2a, left), with a further decline to 78% of control in the more severe type I patient fibroblasts. The *SNCA* mRNA level (Fig. 2a, right) showed a similar decline of 38% from control to SMA type III patient, which decreased further to 83% of control in type I patient fibroblasts. Regression analysis using the RT-PCR cycle counts indicated a significant proportion of the variance in *SNCA* can be explained by knowing the *SMN1* level ($R^2 = 0.49$ ($F(1,7) = 8.8049$, $p < 0.02$)).

NSC-34 cell clones developed to bear differing levels of SMN expression were compared to control cells for *Smn* and *Snca* RNA expression. Clone lines C-6 and C-2 at passage 7, as well as control clones were compared (Fig. 2b). The C-6 clone cells *Smn* expression was 20% lower than the control cells, while *Snca* expression was 48% lower. *Smn* expression in the more severe C-2 clone cells was lowered by 85% compared to control, while *Snca* expression was 98% lower than that of control.

qRT-PCR of SMN and SNCA in Spinal Cord from SMA Type I and Control Patients

Spinal cord RNA samples from SMA type I patients (Fig. 3 left panel, $n = 3$) showed lower *SMN* expression levels as compared to the controls bearing normal *SMN1* dosage ($n = 3$). *SNCA* gene expression levels were also much lower in the same SMA type I patient spinal cord samples (Fig. 3 right panel, $n = 3$). *SMN* was significantly downregulated by 84% in spinal cord samples of SMA type I patients as compared to non-SMN-deficient controls ($n = 3$, $p < 0.02$). *SNCA* was significantly downregulated by 40% in the same SMA type I patients ($n = 3$, $p < 0.01$).

SNCA Protein Analysis in Cells with Low Smn Levels

The intensity of SNCA 19 kD and its dimer band intensities/ β -actin density ratios were compared in cells with lowered SMN levels. Other isoforms and likely polymerized SNCA immunoreactive bands were obtained, but not quantified. Samples from NSC-34 cells, *Smn*-depleted NSC-34 clone lines C-6 and C-2 at passage 7, as well as NSC-34 cells transfected 72 h previously with a control empty vector or a plasmid bearing shSMN, as well as human fibroblasts from controls, SMA type III patient, the mother of an SMA type I patient, and the SMA type I patient are shown (Fig. 4). The bands produced using the polyclonal SNCA antibody produced a weaker monomer band and a more evident dimer, the latter of which is shown here at about 35 kDa. The data for each are reported here as monomer/dimer. Consistent with the RT-PCR data in Fig. 2b, the C-6 and the C-2 NSC clone cell SNCA protein levels were lowered by 49/32(monomer/dimer)% and 80/49%, respectively, as compared to the control NSC clone cell line (Fig. 4, left bands). Similarly, transient SMN downregulation in NSC-34 cells lowered SNCA protein by 62/31% measured at 72 h after transfection compared to control transfected cells (Fig. 4, center bands). SNCA protein in fibroblasts from an SMA type III patient was lowered by 35/26% compared to controls (Fig. 4, right bands). Fibroblasts from the mother of a type I patient had a level 35/33% lower than control, while the type I patient SNCA levels were 77/58% lower than control fibroblasts. These results are representative of at least three different running of the blot and correspond closely with a previous analysis performed on the same samples with a different antibody to SNCA that was monoclonal (Supp. Fig. 1).

Synaptic Vesicular Genes and Low SMN

A further qRT-PCR examination of the spinal cord RNA samples from SMA type I patients and NSC-34 cell clones bearing differing levels of SMN expression was performed to test the specificity of the effects of low *Smn*. Synaptic vesicle protein 2A (SV2A, (Yao et al.;

Bajjalieh et al. 1992)) and synapsin-2 (SYN2, (Thiel et al. 1990; Samigullin et al. 2004)) have both been associated with vesicular processes in motor neurons. Spinal cord RNA samples from two different type I SMA patients showed lower expression of both genes as compared to the control spinal cord sample (Fig. 5a). Both genes also showed a profile of decreasing RNA expression in NSC-34 cells with lowered SMN. The milder C-6 and more severe C2 cells data suggest a graded decrease in Sv2a and Syn2 expression relative to controls.

Discussion

Cell culture models for SMA developed using RNA interference to lower SMN levels resulted in a novel finding that alpha-synuclein expression was significantly lower in neuronal cells with low SMN levels. Furthermore, fibroblast cells derived from SMA patients also showed lower SNCA transcript and protein levels compared to fibroblasts from an SMA carrier or control individual.

SMN levels and SNCA levels show a significant decrease in spinal cord samples from SMA type 1 patients. Prior assessment of both brain and spinal cords in samples from the same patients showed the same significant decrease (Supp. Fig. 2). There is a clear decrease in the levels of both SMN and SNCA as compared to the non-SMN associated motor neuron disorders. Further examination will be necessary to show how they relate across a larger sample selection, across differing severity subtypes, and more interestingly, attempting to correlate SNCA levels with functional tests independent of subtype category, as SNCA may serve as a biomarker in clinical studies.

In neuronal cells, similar to that used in our experiments, it has been demonstrated that downregulation of SNCA had a specific effect on viability, but not proliferative potential (Liu et al. 2008). Elevation of SNCA by inhibition of HDAC increases neuronal protection against death induced by glutamate (Leng and Chuang 2006) or 6-OHDA (Monti et al. 2007). Knockdown of SNCA potentiated 6-OHDA-induced death (Monti et al. 2007). Interestingly, it has previously been suggested that the viability of peripheral sensory neurons which express high levels of SNCA was unaffected by the downregulation of alpha- or gamma-synuclein or both (Papachroni et al. 2005). Axotomy of facial motor neurons, which normally do not express SNCA, elicits an increase in SNCA expression and non-apoptotic cell death (Moran et al. 2001).

Intrinsically disordered proteins such as SNCA may be relevant to multiple neurodegenerative diseases (Uversky 2009). Since SNCA has also been associated with vesicular transport (Utton et al. 2005; Roy et al. 2008) and synaptic function (Withers et al. 1997; Abeliovich et al. 2000; Murphy et al. 2000; Cabin et al. 2002; Utton et al. 2005; Waxman and Giasson 2009), this raises the intriguing question: Does SNCA play a role in the molecular pathology of anterior horn degeneration in SMA? Furthermore, SNCA merits consideration as either a biomarker of, or a functional contributor to, SMA pathogenesis. The relevance of SNCA to other neurodegenerative diseases such as Parkinson's disease (PD) is well-established (Polymeropoulos et al. 1997; Abeliovich et al. 2000; Braak et al. 2000), and brain-specific SNCA alternative splicing has been implicated in Alzheimer's disease (Beyer et al. 2008). The neuroprotective effects of valproic acid in a model of PD have very recently been suggested to be mediated by SNCA (Monti et al. 2010). The recent demonstration of SNCA post-transcriptional repression by a microRNA (Junn et al. 2009) encourages exploration of whether noncoding-RNA-mediated SNCA regulation is a general mechanism in multiple diseases, including SMA.

Finally, the examination of two genes related to neuronal vesicular transport showed a similar though less dramatic decrease in expression of these synaptic vesicular genes. It is of interest that a recent publication (Boon et al. 2009) has also indicated SV2A as an important outcome measure in their investigations of the NMJ in SMA.

Given its association with neuronal synapses, we might well expect SNCA dysfunction to be predictive of impaired neurotransmission and hence more tightly associated with neurological dysfunction than SMN. In comparison, given the proposed association of SMN with axonal transport and chaperone functions, SMN levels may reflect a more generalized axonal decline rather than specifically impaired neurotransmission.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Stable depletion of SMN in neural cells. Murine SMN mRNA sequence (accession: NM_011420) was used to select target regions for shRNA gene silencing. NSC-34 cells were grown, transfected, and stable cell lines were selected and grown. **a** qRT-PCR for *Smn* expression. Assay was performed at day 5 in triplicate, calculated by comparative Ct method with cDNA sample quantification normalized to GAPDH expression. **b** Semi-quantitative analysis of SMN protein by western blot assay. Individual clones showed corresponding lowering of protein consistent with gene levels in **a**. The density of bands was compared to β-actin. **c** Consistency of SMN depletion. Western blot for SMN at passages 5 and 7 relative to NSC control corrected for β-actin is shown. Note: Blots were run on the same gel, but the image has been cropped for clarity. The mRNA and protein analysis of all clones used across the passages examined showed no significant difference in *Smn* expression. Semi-quantitative western blot showed a ratio of SMN/β-actin of 1.56 and 1.50 for C-2, and 1.897 and 1.909 for C-6 across the passages examined

Figure 2.

qRT-PCR of *SMN* and *SNCA* in human SMA fibroblasts and NSC-34 cell clones. **a** qRT-PCR of *SMN* and *SNCA* in human SMA fibroblasts. Fibroblasts derived from an SMA type I and type III patient were compared to control. *SMN1* (left) showed a decreased expression from control to type III to type I, respectively. *SNCA* mRNA level (right) showed a similar decreasing profile across samples from the same patients. Regression analysis using the RT-PCR cycle counts indicated a significant proportion of the variance in *SNCA* can be explained by knowing the *SMN1* level ($R^2 = 0.49$ ($F(1,7) = 8.8049$, $p < 0.02$)). **b** qRT-PCR of *Smn* and *Snca* in NSC-34 cell clones. NSC-34 cell clones with differing SMN expression level, including that used in Table 1, were examined at passage 7. The C-6 cells *Smn* expression was 20% lower than control, while *Snca* expression was 48% lower. *Smn* expression in the more severe C-2 cells was lowered by 85% compared to control, while *Snca* expression was 98% lower. All measurements in this figure represent averaged triplicates

Figure 3. qRT-PCR of SMN and SNCA from SMA type I and control spinal cord. *Left:* spinal cord tissue *SMN* expression levels were significantly downregulated by 84% in SMA type I patients as compared to controls bearing normal *SMN1* dosage ($n = 3$, $p < 0.02$). *Right:* *SNCA* gene expression levels in the same spinal cord samples was significantly downregulated by 40% in the same SMA type I patients ($n = 3$, $p < 0.01$). Both one-tailed t tests

Figure 4.

SNCA protein analysis in cells with low Smn levels. Proteins samples from stable and transient transfected NSC-34 cells and patient fibroblasts were incubated with polyclonal rabbit anti-SNCA and anti-β-actin antibodies. The intensities were measured, and SNCA/β-actin ratios were compared. Intensities for the weaker monomer and stronger dimer band were quantified from the same stable clones used in Fig. 2; NSC-34 cells transfected with a control vector or shSMN plasmid 72 h previously; and patient fibroblasts from controls, an SMA type III patient, the carrier mother of an SMA type I patient, and the patient. This figure is representative of more than three blots that were run for these data

Figure 5. Synaptic vesicular genes in spinal cord of SMA type I patients and NSC-34 cell clones. **a** qRT-PCR of SV2A and SYN2 in human SMA fibroblasts. Fibroblasts derived from a control and two SMA type I patients were compared. Both vesicular transport genes showed lower expression in both type I patients. **b** qRT-PCR of Sv2a and Syn2 in NSC-34 clones. NSC-34 clones with differing SMN level were examined at passage 7. C-6 and C-2 cells data showed differentially decreased expression of Sv2a and Syn2 expression relative to controls. All represent averaged triplicates

Table 1

qRT-PCR array for known genes participating in “neurodegeneration”

Gene	Function	<i>p</i> value	Fold change
<i>Snca</i>	Vesicular transport	<0.0005	−34.72
<i>Slc1a2</i>	Glial glutamate transporter	<0.0025	+2.74
<i>Baiap2</i>	Brain angiogenesis inhibitor	<0.01	−2.01
<i>Gpx1</i>	Glutathione peroxidase	<0.01	−2.19
<i>Hap1</i>	Huntingtin-associated protein	<0.01	−2.21

Total cellular RNA was extracted from the NSC-34 C-2 cell clone with low SMN and compared to a control clone using a qRT-PCR plate array (StellarArray™ Gene Expression Plate “neurodegeneration” NE0100-MM96; Bar Harbor BioTechnology). Data normalization and analysis were carried out according to global pattern recognition analysis tool. Genes listed had more than twofold change in expression