ORIGINAL ARTICLE

Genetic inhibition of JNK3 ameliorates spinal muscular atrophy

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Abstract

Mutation of the *Survival Motor Neuron 1* (SMN1) gene causes spinal muscular atrophy (SMA), an autosomal recessive neurodegenerative disorder that occurs in early childhood. Degeneration of spinal motor neurons caused by SMN deficiency results in progressive muscle atrophy and death in SMA. The molecular mechanism underlying neurodegeneration in SMA is unknown. No treatment is available to prevent neurodegeneration and reduce the burden of illness in SMA. We report that the c-Jun NH₂-terminal kinase (JNK) signaling pathway mediates neurodegeneration in SMA. The neuron-specific isoform JNK3 is required for neuron degeneration caused by SMN deficiency. JNK3 deficiency reduces degeneration of cultured neurons caused by low levels of SMN. Genetic inhibition of JNK pathway *in vivo* by *Jnk3* knockout results in amelioration of SMA phenotype. JNK3 deficiency prevents the loss of spinal cord motor neurons, reduces muscle degeneration, improves muscle fiber thickness and muscle growth, improves motor function and overall growth and increases lifespan of mice with SMA that shows a systemic rescue of phenotype by a SMN-independent mechanism. JNK3 represents a potential (non-SMN) therapeutic target for the treatment of SMA.

Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder of the early childhood caused by mutation of telomeric copy of the *Survival Motor Neuron 1* (SMN1) gene (1). The SMN2, centromeric copy, undergoes alternative splicing and predominantly produces truncated product lacking exon 7 (SMNΔ7) (2). Low levels of full-length SMN produced by SMN2 are sufficient for embryonic development and survival, but result in the loss of spinal motor neurons leading to muscle atrophy, respiratory failure and death in SMA patients (3).

SMA is a developmental disorder with early onset and stable course, characterized by skeletal muscle atrophy due to the degeneration of spinal motor neurons caused by low levels of SMN (4). Currently, no treatment is available to prevent degeneration of motor neurons in SMA. The cellular and molecular mechanisms of motor neuron degeneration caused by SMN

deficiency are unknown. Progress has been made towards understanding biochemical function of SMN but its role in survival and maintenance of motor neurons is unclear (5). The function of SMN is indicated in the development and maintenance of the nervous system, maturation of neuromuscular junctions (NMJs) and growth of skeletal muscle (4,6–8). SMN is shown to play a role in the assembly of spliceosomal small nuclear ribonucleoproteins (snRNPs) required for pre-mRNA splicing (9). The SMN-dependent splicing defects at pre-, early- and late-symptomatic stages in selective genes (10–12), the SMN-dependent alteration in the levels of different proteins (10,13) and the snRNP biogenesis-independent functions (14) indicate the complex nature of biochemical alterations. These alterations may affect cellular processes such as axonal growth, pathfinding, NMJs, cytoskeleton, synaptic maturation and neurotransmitter release in different neuronal and muscle cells that might be collectively

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responsible for degeneration of spinal motor neurons (5,7). It is possible that the two cellular processes in neurons might be affected simultaneously by the loss of SMN function, (a) reduced efficiency of splicing that may result in low levels of proteins required for survival and maintenance of neurons and (b) activation of intracellular stress signaling pathways that initiate neurodegeneration. The low levels of SMN are the cause of neurodegeneration, but it is unclear why selectively lower spinal cord motor neurons degenerate (5,7). Intracellular signaling mechanisms triggered by the low levels of SMN that might mediate neurodegeneration in SMA are also unclear. Recent studies have indicated that the activation of RhoA/ROCK pathway by SMN deficiency might contribute to the disruption of actin cytoskeleton by hyperphosphorylation of profilin and affect neuron integrity (15,16). Inhibition of Rho kinase is shown to increase survival of SMA mouse model with intermediate severity (17). Alteration in the β -catenin signaling due to reduced expression of ubiquitin-like modifier activating enzyme 1 (UBA1) and increased β -catenin levels might contribute to motor neuron pathology in SMA (13). However, the function of non-SMN targets in fully systemic rescue of SMA pathology without altering levels of SMN (SMN-independent) remains to be examined.

In this study, we investigated the molecular mechanism of neurodegeneration in SMA. We report that the c-Jun NH₂-terminal kinase (JNK) cascades ASK1/MKK4/7/JNK and MEKK1/MKK4/7/JNK are activated in spinal cords of SMA patients and SMA mice and mediate neurodegeneration in SMA. We identified that JNK3, a neuron-specific isoform, mediates neurodegeneration caused by the low levels of SMN. Deficiency of JNK3 (*Jnk3*^{-/-}) resulted in reduced degeneration of cultured neurons with low levels of SMN. To examine the effect of JNK3 deficiency *in vivo*, we created SMA carrier mice lacking JNK3 by crossing SMA carrier mice with *Jnk3*^{-/-} knockout mice [*Jnk3*^{-/-} mice display normal phenotype, including fertility and lifespan (18,19)]. Genetic inhibition of JNK pathway *in vivo* by *Jnk3* knockout resulted in amelioration of SMA phenotype. JNK3 deficiency results in systemic rescue of phenotype without altering levels of SMN (SMN-independent) by preventing degeneration of spinal cord motor neurons, reducing muscle atrophy, improving overall growth and increasing lifespan of mice with SMA. We propose that the JNK3 represents a potential (non-SMN) therapeutic target for the treatment of SMA.

Results

Activation of the JNK signaling pathway in SMA

The molecular mechanism of neurodegeneration caused by low levels of SMN in SMA is unknown. To identify signaling mechanisms that might mediate neurodegeneration in SMA, we examined the phosphorylation of mitogen activated protein kinases (MAPKs) in the spinal cords (lumbar region, *area of primary pathogenesis in SMA*) from non-SMA human (Normal, #83, age 69 days) and SMA (type-I) patients (i) SMA4583, age 77 days (ii) SMA4994, age 59 days and (iii) SMA4629, age 169 days and from 12-day old non-SMA and mice with SMA-like disease (SMA mice) (20). We performed biochemical experiments using individual SMA patient samples and by pooling SMA patient samples to reduce the biological variation. Averaging of data from three individual patient samples and pooling of three SMA patient samples resulted in similar activity. We compared quantification and statistical significance (one-way analysis of variance, ANOVA) of data for SMA mice and SMA patients from two different MAPK arrays and focused our analysis on kinases that were commonly activated in both SMA patient and SMA mice.

The phospho-MAPK array analysis of human spinal cord samples shows an increase in phosphorylation of almost all MAP kinases except MKK3 and it was not possible to identify specific kinases activated in SMA (Fig. 1A). The non-specific increase in phosphorylation of kinases might be because of longer post-mortem interval (PMI) for processing human tissues (avg. ~8.5 h). To gain insight into the activation of specific MAP kinases in SMA, we examined spinal cord samples from normal and SMA mice (PMI, ~30 min) and compared these with the human samples (Fig. 1B) to identify kinases commonly activated in mouse and human spinal cord. Comparative analysis of data from phospho-MAPK antibody array of human and mice spinal cord samples, shows that several MAP kinases (AKT1-3, ERK2, MKK3, MKK6, p38 α , p38 β , p38 γ , p38 δ , p70, RSK1-2 and TOR) were not commonly activated in mice and human samples (Fig. 1A and B), and these kinases were not considered for further analysis. However, three groups of MAP kinases (i) JNK group (JNK1, JNK2, JNK3), (ii) glycogen synthase kinase 3 (GSK-3 α and β) and (iii) extracellular signal-regulated kinase (ERK1) showed increased phosphorylation in both SMA patients and SMA mice compared with normal (Fig. 1). Quantitative analyses showed moderate increase in phosphorylation of GSK-3 α/β ($10.10 \pm 1.09\%$, $P = 0.086$) in SMA mice and SMA patients ($39.29 \pm 4.48\%$, $P = 0.037$), but the increase is not statistically significant in SMA mice. The increase in phosphorylation of GSK-3 β in SMA patients ($25.94 \pm 14.12\%$, $P = 0.304$) and SMA mice ($2.45 \pm 0.23\%$, $P = 0.10$) was not considered significant because of P -value > 0.05 . Increase in phosphorylation of ERK1 ($43 \pm 2.42\%$, $P = 0.001$) in SMA patients and SMA mice ($83.12 \pm 1.13\%$, $P = 0.000$) were significant. In contrast, increase in phosphorylation of ERK2 was not detected in SMA mice and indicates better specificity with reduced PMI (Fig. 1). Interestingly, increase in phosphorylation of all JNK isoforms JNK1, JNK2 and JNK3 was detected in both SMA patients and SMA mice (Fig. 1A and B). The suppression of Akt activity in SMA mice indicates activation of JNK-mediated neuronal death (Fig. 1B) (21). The total increase in phosphorylation of JNK (JNK pan) in mouse ($39 \pm 3.2\%$, $P = 0.004$) and in human ($34 \pm 3.1\%$, $P = 0.017$) was statistically significant and indicated possibility of JNK activation in SMA. Further, Bonferroni's correction for multiple comparisons (mouse) among activation of kinases ERK ($P < 0.001$), GSK ($P < 0.001$) and JNK ($P < 0.0001$) signaling pathways suggest that activation of the JNK pathway is most significant and supports JNK activation in SMA.

To further test activation of JNK and to gain insight into upstream molecular components of the JNK signaling cascade, we employed second phospho-MAPK antibody array (Full-Moon Bio-Systems). We used standard and custom designed arrays to identify molecular targets that might be activated by intracellular stress caused by low levels of SMN in SMA. Total change in phosphorylation represents averaging of data of three SMA patients (Fig. 2C). The significance (P -value) of change in the levels of phosphorylation was determined by one-way ANOVA analysis (mean $\pm$ s.e.m., $n = 6$). Phospho-MAPK analysis did not show marked change in phosphorylation of GSK-3 α/β and p38 MAP kinase in SMA mice (Fig. 2A). The small increase in levels of phospho-GSK-3 α/β ($P = 0.193$) and phospho-p38 ($P = 0.20$) in SMA patient samples was not significant (Fig. 2A). The increase in the levels of p-JNK and p-ERK1 (Fig. 2A–C) in SMA patients and SMA mice is consistent with the results of first MAPK array (Fig. 1).

To test whether increase in phosphorylation of ERK1 and JNKs is associated with activation of these kinases, we examined phosphorylation of targets of JNK (c-Jun, JunB, JunD and Elk-1) and ERK1 (ATF-1, Elk-1 and JunD) (22). Comparison of normal and SMA mice did not show significant change ($P = 0.94$) in levels

Figure 1. Activation of JNK group of MAP kinases in SMA. Analysis of activation of protein kinases in spinal cord tissues by phospho-MAPK antibody array (R&D Systems). (A) Spinal cord protein extracts from age-matched non-SMA (Normal, stripes) and combination of three SMA patients (4583 + 4629 + 4994) (texture) were examined. Changes in the phosphorylation of kinases are presented as [mean $\pm$ s.e.m. Control $n=1$, SMA $n=3$ with four replicates per sample ($n=4$)]. ***Three SMA patient samples were pooled and examined against single (non-SMA) control. (B) Protein extracts from spinal cords of 12-day old non-SMA (Normal, grey) and with SMA-like disease (SMA, black) littermates were examined. Changes in the phosphorylation of kinases are presented [mean $\pm$ s.e.m., 3/mice per group with four replicates per group ($n=4$)] as bar graphs. Insets show IBs for SMN and tubulin. Statistical significance (P -values) of data was calculated by one-way ANOVA. Total JNK (JNKpan) activation P -value. Asterisks (*) indicate kinases activated in both mice and human.

of p-JunD (Fig. 2D). Small increase in p-ATF-1 (~1.4-fold) in mice ($P=0.016$) and human ($P=0.022$) and p-Elk-1 (~1.3-fold) in mice ($P=0.012$) and human ($P=0.068^{**}$, **not significant) suggest mild ERK1 activation. Increase in phosphorylation of ERK1 and Elk-1 is reported in spinal cords of severe SMA mice and the activation of MEK1/ERK1/Elk-1 cascade may play a role in negative regulation of SMN expression (23). However, JNK can also contribute to phosphorylation of Elk-1 (24). Interestingly, comparison of phospho-c-Jun (p-c-Jun) in normal and SMA spinal cords shows substantial increase in levels of p-c-Jun in SMA mice (2.47-fold, $P=0.000$) and human (3.01-fold, $P=0.003$) SMA patients (Fig. 2D–F). These data indicate higher activity of JNK in SMA.

The examination of upstream MAP kinase kinase (MAP2K), MKK4 and MKK7, which are known to activate JNK (25), shows that both MKK4 and MKK7 were activated in SMA mice and patients (Fig. 2A and B). Comparison of p-MKK4 and p-MKK7 indicate that MKK4 activation is higher (4.8-fold, $P=0.007$) than MKK7 (2.6-fold, $P=0.009$) in SMA patients (Fig. 2C) and is consistent with data from SMA mice (Fig. 2A). To identify upstream kinases (MAP3Ks) that may activate MKK4/7, we examined

activation of ASK1 (apoptosis signal-regulating kinase-1) and MEKK1 (MAP3K1) that are known to activate MKK4 and MKK7 (26). Increase in p-ASK1 in mouse (1.7-fold, $P=0.005$) and human (2.0-fold, $P=0.005$) and increase in p-MEKK1 in SMA patients (1.6-fold, $P=0.015$) indicates that ASK1 and MEKK1 may contribute to JNK activation (Fig. 2A and B). These data indicate that the two signaling modules, (i) ASK1/MKK4/7/JNK and (ii) MEKK1/MKK4/7/JNK, may be activated in SMA.

To validate MAPK array data and confirm activation of the JNK pathway in SMA, we examined activation of the JNK signaling components in the spinal cords of mice with SMA using immunoblot (IB) analysis. Quantitative analysis of IBs shows 2.6-fold increase in the levels of p-JNK ($P=0.003$) in SMA mice compared with normal mice (Fig. 3A and B). Analysis of JNK activation in the spinal cords during postnatal development at ages of 2-, 6- and 12-days from SMA mice show an increase from (1.86 ± 0.07 , $P=0.023$)-folds at 2-days to (2.45 ± 0.41 , $P=0.026$)-folds at 6-days and (2.56 ± 0.16 , $P=0.026$)-folds at 12-days of age compared with normal mice (Fig. 3C). The analysis of upstream activators ASK1, MEKK1, MKK4 and MKK7 shows that these kinases are

Figure 2. Activation of upstream MAP kinases that mediate JNK activation in the spinal cords from SMA mice and SMA patients. Analysis of activation of kinases and transcription factors in the spinal cord tissues by phospho-MAPK antibody array (Full-Moon Biosystems). (A) Protein extracts from spinal cords of 12-day old non-SMA (Normal, blue) and SMA mice littermates (red) were examined. Phosphorylation intensities are presented [mean $\pm$ s.e.m., 3/mice per group with six replicates per group ($n = 6$)] as bar graphs. (B) Spinal cord protein extracts from age-matched non-SMA (Normal, blue) and three SMA patients, SMA4583 (red), SMA4629 (green), SMA4994 (violet) were examined. Insets in (a) and (b) show IBs for SMN and tubulin. (C) Averaged phosphorylation intensities of MAP kinases from three individual SMA patients (SMA4583, SMA4629, SMA4994) (SMA, orange) and non-SMA (Normal, blue) presented [mean $\pm$ s.e.m., Control $n = 1$, SMA $n = 3$ with six replicates per sample ($n = 6$)] as bar graphs. (D–F) Analysis of down stream targets (transcription factors) of activated MAP kinases using phospho-protein antibody array. (D) Protein extracts from the spinal cords of 12-day old non-SMA (Normal, blue) and SMA mice littermates (red) were examined. (E) Protein extracts from the spinal cords of non-SMA (Normal, blue) and three SMA patients, SMA4583 (red), SMA4629 (green), SMA4994 (violet) were examined. Phosphorylation intensities of individual human subjects (mean $\pm$ s.e.m., $n = 6$) plotted as bar graph. (F) Averaged phosphorylation intensities of transcription factors from three individual SMA patients [SMA4583, SMA4629, SMA4994] (SMA, orange) and non-SMA (Normal, blue) are presented [mean $\pm$ s.e.m., Control $n = 1$, SMA $n = 3$ with six replicates per sample ($n = 6$)] as bar graph. Statistical significance (P-values) of data was calculated by one-way ANOVA. *P-value not significant. Asterisks (*) indicate kinases activated in both mice and human with P-value < 0.05 .

also phosphorylated and activated in SMA mice at 2-days and 6-days of age (Supplementary Material, Fig. S1), and these data are consistent with 12-days age SMA mice (Fig. 3A). These data

suggest an early activation of JNK in SMA mice that continues to increase with age and might contribute to increasing severity of SMA disease. We examined JNK downstream targets, ATF-2

Figure 3. Immunoblot analysis of activation of the JNK signaling cascade in SMA mice and SMA patients. (A) Activation of the JNK pathway in SMA mice. Protein extracts from the spinal cords of 12-day old non-SMA (Normal) and with SMA-like disease (SMA) littermates were prepared and examined by IB analysis using antibodies against SMN, kinases, phospho-kinases and α -tubulin. Two spinal cords pooled together for each non-SMA (Normal) and SMA-S1 (Set1) littermates and SMA-S2 (Set2) from another litter. (B) The levels of phospho-proteins (p-c-Jun, p-JNK, p-MKK4, p-MKK7, p-MLK3) in the spinal cord samples from Normal (grey) and SMA (black) mice were determined by quantitative analysis of IBs, normalized against non-phospho proteins and represented as a bar graph (mean $\pm$ s.e.m.; Control $n = 2$, SMA $n = 4$). (C) JNK activation during postnatal development of SMA mice. Immunoblot analysis of the spinal cord tissues from 2-, 6- and 12-days old Normal and SMA littermates using antibodies against JNK and phospho-JNK (p-JNK). The relative levels of p-JNK in Normal and SMA littermates were normalized to total JNK and presented as fold change (mean $\pm$ s.e.m., $n = 2$) in a bar graph. Asterisks (*) indicate P-value is < 0.05 . (D) Immunoblot analysis of the brain tissue protein extracts from non-SMA (Normal) and mix of three SMA patients (4583 + 4629 + 4994) using antibodies against kinases, phospho-kinases and α -tubulin. (E) The levels of phospho-MAP kinases in the brain samples from Normal (stripes) and SMA patients (texture) mice were determined by quantitative analysis of IBs, normalized against respective non-phospho protein and presented as bar graph (mean $\pm$ s.e.m.; Control $n = 1$, SMA $n = 3$ with two replicates $n = 2$). Statistical significance (P value) of activation of kinases was determined by Student's t-test (unpaired) with Welch's correction. P-values indicated for each kinase on the top of bar graphs.

and c-Jun. No change in phosphorylation of ATF-2 was detected. Notably, significant increase in the levels of phospho-c-Jun (3.6-fold, $P = 0.004$) was detected in the spinal cords of SMA compared with normal mice (Fig. 3A and B). Increase in phospho-JNK and phospho-c-Jun levels in the spinal cords of SMA mice compared with normal mice supports *in vivo* activation of JNK in SMA. A small increase ($20 \pm 3.0\%$) in the levels p-MKK7 was detected in SMA compared with normal mice (Fig. 3A and B). Interestingly, a large increase (12.5-fold, $P < 0.0001$) in the levels of p-MKK4 was detected in SMA compared with normal mice (Fig. 3A and B). These data are consistent with the MAPK array and suggest that both MKK4 and MKK7 might contribute to JNK activation.

Further we examined MAP3Ks, MLK3 (mixed-lineage kinase3), ASK1 and MEKK1 (MAP3K1) that are known to activate MKK4 and

MKK7 (25). No change in levels of phospho-MLK3 was detected (Fig. 3A and B). Increase in p-MEKK1 (1.6-fold, $P = 0.015$) levels in the spinal cords of SMA mice compared with normal mice (Fig. 3A and B) is similar to increase observed in SMA patients (Fig. 2C). Interestingly, 2.0-fold increase ($P = 0.001$) in the levels of p-ASK1 (Fig. 3A and B) indicates that both MKK4 and MKK7 may also be activated by ASK1. These data supports that two signaling modules mediated by ASK1 and MEKK1 may contribute to JNK activation.

The examination of SMA patient brain tissues shows activation of the JNK pathway ASK1/MEKK1 $\rightarrow$ MKK4/7 $\rightarrow$ JNK (Fig. 3D and E) that is similar to MAPK array data of the spinal cord from SMA patients (Fig. 2), and is consistent with data of the spinal cords from SMA mice. These data suggests that low levels

of SMN result in activation of JNK signaling *in vivo* under physiological conditions associated with the pathogenesis of SMA.

JNK activation in the spinal cord motor neurons from SMA mice

The activation of JNK in the spinal cords of SMA patient and SMA mice suggest that JNK might be activated in the spinal cord motor neurons. To examine JNK activation in motor neurons, we cultured spinal cord motor neurons from 7-day old normal and SMA littermates as described previously (27), and examined phosphorylation of JNK and its target c-Jun (28). We examined two types of neurons from the spinal cord cultures, fully differentiated motor neurons and partially differentiated spinal cord neurons with motor neuron-like characteristics that might be generated from endogenous population of neural progenitor cells and/or stem cells of the spinal cord. The double staining of the spinal cord neurons with neuron-specific β -tubulin (class-III) and motor neuron markers, choline acetyltransferase (ChAT) or Homeobox b9 (Hoxb9 or Hb9) show that the majority of neuron-specific β -tubulin stained neurons are Hb9 and ChAT positive (Supplementary Material, Fig. S2). The *in vitro* growth and differentiation of spinal cord motor neurons from SMA mice were severely impaired compared with neurons from

normal (non-SMA) mice (Fig. 4). In addition to growth defects, marked neuronal degeneration, including axonal thickening, retraction, swelling and degeneration was evident in neurons from SMA mice compared with normal mice and is consistent with previous findings (Fig. 4) (27,29,30). Immunofluorescence analysis shows increased phosphorylation of c-Jun (p-c-Jun, Fig. 4A and C) and activation of JNK (p-JNK, Fig. 4B and D) in spinal cord motor neurons from SMA mice compared with normal mice. These data show that the JNK is activated in spinal cord motor neurons from SMA mice and is consistent with our data from the total spinal cord protein extracts from SMA mice and SMA patients.

JNK3 mediates neuron degeneration caused by SMN deficiency

The JNK group of MAP kinase is encoded by the *Jnk1*, *Jnk2* and *Jnk3* genes. The *Jnk1* and *Jnk2* genes are ubiquitously expressed. In contrast, *Jnk3* is mainly expressed in the CNS, with some expression in the heart and testis (26). MAPK array analysis of total spinal cord tissue shows activation of all JNK isoforms in both SMA mice and human patients (Fig. 1). It is possible that all JNK isoforms are phosphorylated because spinal cord contains different cell types, including neurons and glial cells. However, it is unclear which JNK isoform is required for neuron degeneration

Figure 4. Activation of JNK in the spinal cord motor neurons from SMA mice. (A) The spinal cord ex-plants were cultured *in vitro* for 12 days to grow motor neuron from 7-day old normal and SMA mice, fixed with 4% PFA and stained with antibodies to neuron-specific β -tubulin class-III (red) and p-c-Jun (green). Scale bar is 50 μ m. (B) Motor neurons stained with antibodies to β -tubulin (red) and p-JNK (green). Scale bar is 50 μ m. (C) The spinal cord ex-plants were cultured *in vitro* for 12 days to grow and differentiate neurons with motor neuron-like characteristics including positivity for motor neuron markers, ChAT and Hoxb9 (see Supplementary Material, Fig. S1) from 7-day old normal and SMA mice, fixed with 4% PFA and stained with antibodies to neuron-specific β -tubulin class-III (red) and p-c-Jun (green). Scale bar is 20 μ m. (D) Neurons stained with antibodies to β -tubulin (red) and p-JNK (green). Nuclei are stained with DAPI (blue). Arrows indicate axonal swelling, thickening and degeneration. Arrowheads show accumulation of activated p-JNK or p-c-Jun in the nucleus. Scale bar is 20 μ m.

caused by low levels of SMN. To identify the specific JNK isoform required for neurodegeneration, large numbers of purified neurons were required for *in vitro* biochemical experiments. Therefore, we used purified cultures of primary cerebellar granule neurons (CGNs) from 7-day old mice (31), and examined phosphorylation of JNK and its target c-Jun to establish that JNK is activated by SMN deficiency in cultured neurons. First, we tested whether JNK is activated in cultured CGN from SMA mice. Primary CGN from the normal and SMA mice 7-day old littermates were cultured and stained with antibodies against phospho-c-Jun and phospho-JNK. The growth of cultured neurons from SMA mice was severely impaired and gradual degeneration was noticed compared with neurons from normal littermates (Supplementary Material, Fig. S3). Immunofluorescence analysis shows phosphorylation of c-Jun (Supplementary Material, Fig. S3A) and activation of JNK (Supplementary Material, Fig. S3B) in neurons from SMA mice compared with normal mice. These data show that the JNK is also activated in purified cultures of CGN from SMA mice, and is consistent with data from cultured spinal cord motor neurons. These data suggest that the low levels of SMN associated with SMA might not support normal growth of neurons from SMA mice under *in vitro* experimental conditions.

To generate sufficient amount of neurons, we cultured purified neurons (CGN) from normal mice and developed cell-based model to knockdown SMN expression using RNA interference (RNAi). Neurons were transfected with Scramble II siRNA (Control) or *Smn* specific siRNA (siRNA-*Smn*) to knockdown SMN levels using RNAi. Knockdown of *Smn* expression reduced SMN levels by $68.06 \pm 2.52\%$ (Fig. 5A and B). Low levels of SMN ($\sim 30\%$) in neurons (siRNA-*Smn*) resulted in axonal degeneration and the loss of neurons compared with control neurons (Fig. 5C). The phospho-c-Jun was not detected in control neurons treated with scrambled siRNA (Fig. 5D, Control). In contrast, neurons treated with siRNA-*Smn* show robust phosphorylation of c-Jun (Fig. 5D, siRNA-*Smn*). Comparison of SMN-deficient neurons to controls shows marked increase in staining of phospho-JNK in degenerating axons and accumulation in the nucleus (Fig. 5E). Nuclear accumulation of p-c-Jun and p-JNK indicates activation of JNK in cultured primary neurons by low levels of SMN and suggests that CGN could be used for examining effects of SMN deficiency.

To identify specific JNK isoform that mediate neurodegeneration caused by SMN deficiency, we used purified cultured CGN treated with siRNA-*Smn*, and examined with the MAP kinase array. The MAPK array shows a marked increase (10-fold) in the

Figure 5. SMN deficiency causes JNK activation in cultured primary neurons. (A) The *Smn* gene suppression causes axonal defects in primary CGNs from 7-day old mice. Neurons were cultured *in vitro* for 6-days, mock-transfected (control), transfected with scrambled siRNA (Scramble) or siRNA-*Smn*. Levels of expression of SMN, and tubulin (72 h post transfection) were examined by IB analysis using specific antibodies. (B) The relative levels of SMN in cultured neurons Control (mock-transfected), siRNA-*Smn* (transfected with siRNA-*Smn*) and Scramble (transfected with scrambled siRNA) were normalized to tubulin and presented as % change (mean $\pm$ s.e.m., $n = 3$) in a bar graph. (C) CGNs isolated from 7-day old mice, transfected with scrambled (Control) or siRNA-*Smn* were cultured for 72 h, fixed and stained with antibodies to neuron-specific, class-III β -tubulin (red) and SMN (green). (D) SMN deficiency causes phosphorylation of c-Jun. Neurons were stained with antibodies to β -tubulin (red) and phospho-c-Jun (p-c-Jun, green). (E) SMN deficiency causes activation of JNK in neurons. Neurons were stained with antibodies to β -tubulin (red) and phospho-JNK (p-JNK, green). Nuclei stained with DAPI (blue). Stained neurons were examined by confocal microscopy. Scale bar is 8.0 μ m.

phosphorylation of JNK3, a neuron-specific isoform (19), in degenerating neurons caused by SMN knockdown (Fig. 6A). These data indicate that the reduced levels of SMN cause preferential activation of JNK3 in neurons. To test whether JNK3 is required for neurodegeneration, we examined the effect of SMN deficiency on neurons lacking JNK3. Neurons from wild-type *Jnk3*^{+/+} and knockout *Jnk3*^{-/-} mice were cultured and transfected with siRNA. Knockdown of SMN resulted in activation of JNK (p-JNK) and degeneration of *Jnk3*^{+/+} neurons (Fig. 6B, Tubulin). Interestingly, p-JNK was not detected in *Jnk3*^{-/-} neurons lacking SMN and degeneration of *Jnk3*^{-/-} neurons was reduced compared with

Jnk3^{+/+} neurons (Fig. 6B). To further test whether JNK3 is required for degeneration of SMN-deficient neurons, we complemented *Jnk3*^{-/-} neurons with recombinant GFP-JNK3 (32) using transient transfection, and examined the effect of SMN deficiency. Control experiments show GFP-JNK3 was distributed in the axons and soma, but excluded from the nucleus of *Jnk3*^{-/-} neurons. The absence of GFP-JNK3 in the nucleus of healthy neurons indicates that the JNK3 is not activated (Fig. 6C). Interestingly, knockdown of SMN results in degeneration of *Jnk3*^{-/-} neurons expressing GFP-JNK3. The accumulation of GFP-JNK3 in the nucleus of degenerating neurons indicates phosphorylation and activation of JNK3 (Fig. 6C). These data suggest that JNK3 contributes to neurodegeneration caused by SMN deficiency. Comparison of neurodegeneration caused by SMN knockdown in *Jnk3*^{+/+} and *Jnk3*^{-/-} neurons shows JNK3 deficiency provides neuroprotection ($57.97 \pm 0.92\%$, $P < 0.0001$), and about 80% of the neurons survive (Fig. 6D). Together, these data show that JNK3 is important for neurodegeneration caused by SMN deficiency and genetic elimination of JNK3 provides neuroprotection. These findings suggest that JNK may be a potential target to reduce neurodegeneration and may help rescue SMA phenotype.

Figure 6. Neuron-specific isoform JNK3 is required for neuron degeneration caused by SMN deficiency. (A) SMN deficiency causes activation of JNK3, a neuron-specific isoforms, in cultured primary neurons. Cultured mouse CGNs were transfected with scrambled siRNA (Control) or siRNA-Smn. The levels of phosphorylation were examined by phospho-MAPK antibody array (R&D Systems Inc.). The levels of phosphorylation of individual JNK isoforms, JNK1 JNK2 and JNK3 were quantitated by densitometry and relative intensities were calculated (mean $\pm$ s.e.m., $n = 4$) to plot fold activation as a bar graph. (B) JNK3 is required for neuron degeneration caused by SMN deficiency. JNK3 deficiency reduces neuron degeneration caused by low levels of SMN. Neurons (CGN) from wild-type (*Jnk3*^{+/+}) and knockout (*Jnk3*^{-/-}) 7-day old mice transfected with scrambled (Control) or siRNA-Smn were cultured for 72 h and stained with antibodies to β -tubulin (red) and phospho-JNK (green). (C) Neurons from knockout *Jnk3*^{-/-} mice were transfected with plasmid expressing GFP-JNK3 and with either scrambled (Control) or siRNA-Smn, cultured for 72 h and stained with antibodies to β -tubulin (red). JNK3 was examined by GFP fluorescence (green) using confocal microscopy. Nuclei stained with DAPI (blue). Scale bar is 20 μ m. (D) Quantification of neuron degeneration. Neurons with intact axons were counted in Control (transfected with scrambled), siRNA-Smn (treated with SMN specific siRNA) and *Jnk3*^{-/-} (neurons lacking JNK3). Thirty images, 10 each from three different experiments for control or treated were examined for neuron degeneration. The average (mean $\pm$ s.e.m.; $n = 3$) percent of surviving neurons stained with nuclear DAPI is plotted as a bar graph. The statistical significance (P-value) of neuroprotection data was determined by one-way ANOVA and unpaired t-test with Welch's correction (two tailed). * $P < 0.0001$.

Genetic inactivation of *Jnk3* rescues SMA phenotype in mice

To test whether *in vivo* *Jnk3* inactivation will help reduce neurodegeneration and rescue phenotype in mice with SMA, we generated SMN Δ 7 carrier mice with *Jnk3* mutation (*Jnk3*^{-/-}) [*Smn*^{-/-}; SMN2^{+/+}; SMN Δ 7^{+/+}; *Jnk3*^{-/-}] to produce littermates with SMA-like disease with *Jnk3*^{+/+} (SMA) and *Jnk3*^{-/-} (SMA-J3) and examined phenotype. The *Jnk3*^{-/-} knockout mice were characterized previously and displayed normal phenotype, including fertility and lifespan; however, JNK3 deficiency reduced neurodegeneration induced by kainic acid-mediated excitotoxicity and ischemia (18,19). Interestingly, JNK3 deficiency resulted in amelioration of SMA phenotype by improving motor function, overall growth and survival of mice with SMA (Figs 7–9). Mice with SMA lacking *Jnk3*^{-/-} (SMA-J3) were healthy, and able to stand and walk compared with SMA mice (SMA) between the ages of 6–10 days (Fig. 7A). Analysis of gross motor functions show JNK3 deficiency improves motor functions of mice with SMA. The ability to stand on four paws and walk (time in sec) was improved in SMA-J3 mice (22.20 ± 3.74 , $P = 0.0063$) compared with SMA mice (2.50 ± 0.20) (Fig. 7B). Analysis of time-to-right (TTR) shows significant improvement ($P < 0.0001$) in the motor function of SMA-J3 mice compared with SMA mice at 7 and 8-days of age (Fig. 7C). To test whether improvement in the phenotype of SMA-J3 mice is independent of changes in the SMN levels, we examined whether JNK3 deficiency has any effect on the levels of SMN. Quantitative analysis of IBs of the spinal cord, brain and muscle tissues did not show change in the levels of SMN protein in SMA-J3 mice compared with SMA mice (Fig. 7D and E). These data suggest that the improvement in SMA phenotype by JNK3 deficiency may be independent of SMN.

Deletion of the *Jnk3* gene resulted in increase of growth-period from 8-days (SMA) to 14-days (SMA-J3), and shows 1.75-fold ($P = 0.0023$) increase in growth-period of SMA-J3 mice compared with SMA mice. SMA-J3 mice gained higher average peak body weight (g) [4.6 ± 0.30] than SMA [2.88 ± 0.32]. The change in body weight (g) from birth to peak for SMA (1.46 ± 0.25) and SMA-J3 (2.98 ± 0.37) shows a 2.05-fold increase in body growth of SMA-J3 mice (Fig. 8A). It is established that the embryonic growth and weight of SMA newborn babies are slow and lower compared with normal babies. To examine whether JNK3 deficiency also improved

Figure 7. Genetic inactivation of JNK3 improves gross motor function in mice with SMA without changing levels of the SMN protein. (A) A photograph of 10-day old littermates generated by breeding of SMA carrier mice SMA-J3^{het} [*Smn*^{-/-}; *SMN2*^{+/+}; *SMNΔ7*^{+/+}; *Jnk3*^{-/-}], non-SMA (Normal) [*Smn*^{+/+}; *SMN2*^{+/+}; *SMNΔ7*^{+/+}; *Jnk3*^{+/+}], SMA [*Smn*^{-/-}; *SMN2*^{+/+}; *SMNΔ7*^{+/+}; *Jnk3*^{+/+}] and SMA mouse with *Jnk3* mutation (SMA-J3) [*Smn*^{-/-}; *SMN2*^{+/+}; *SMNΔ7*^{+/+}; *Jnk3*^{-/-}]. Genotypes were examined by PCR. (B) JNK3 deficiency improves ability of mice with SMA for motor action. Ability to stand on four paws and walk was determined by measuring the time to fall off on four paws in an effort to walk, and was recorded in 8-day old Normal, SMA and SMA-J3 littermates with all three genotypes present in the same litter. Latency to fall (sec) is presented as (mean ± s.e.m., n = 5 mice/group) in a bar graph. Unpaired t-test (two tailed) between SMA and SMA-J3 shows significant ($P = 0.0063$) improvement in the motor ability of SMA-J3 in comparison to SMA mice [dotted line indicates time limit (30 s) of test]. (C) JNK3 deficiency improves gross motor function. Ability to right was recorded for 5, 6, 7 and 8-day old Normal, SMA and SMA-J3 littermates with all three genotypes present in the same litter. TTR with a time limit of 30 s for test and three recordings per pup is plotted as (mean ± s.e.m., n = 5 mice/group). Improvement in the motor function is indicated by significant increase in the ability to right for SMA-J3 mice compared with SMA mice. * (ANOVA, $P < 0.0001$; t-test $P = 0.0001$) ** (ANOVA, $P < 0.0001$; t-test $P = 0.0038$). (D) JNK deficiency did not alter *in vivo* levels of SMN protein in SMA-J3 mice. Immunoblot analysis of the brain, spinal cord and skeletal muscle tissues using antibody against SMN from 8-day old Normal, SMA and SMA-J3 littermates with all three genotypes present in the same litter. (E) The relative levels of SMN in the brain, spinal cord and skeletal muscle tissues from Normal, SMA and SMA-J3 littermates were normalized to α -tubulin and presented as relative (%) (mean ± s.e.m., n = 3) in a bar graph.

embryonic growth, we compared weights of Normal (1.76 ± 0.031), SMA (1.42 ± 0.047) and SMA-J3 (1.63 ± 0.052) mice at birth. The reduced weight loss in newborn pups of SMA-J3 (0.13 g) than SMA (0.34 g) compared with Normal mice shows a 2.62-fold improvement in embryonic growth of SMA mice lacking JNK3 (ANOVA, $P < 0.0001$). These data suggest that JNK3 deficiency improves embryonic and postnatal growth of mice with SMA. Mutation of *Jnk3* also resulted in 2-fold increase in average survival of SMA-J3 mice (14.95 ± 0.79) days compared with SMA (7.14 ± 0.93 , Log-rank test, $P < 0.0001$). Notably, the 7 days increase in maximum life of SMA-J3 mice (45% increase in lifespan) includes 6 days of increase in growth (reduced severity) that shows about 86% of the total increase in lifespan is with reduced burden of illness. Importantly, the 4.20-fold increase in initial survival of SMA-J3 mice compared with SMA mice suggests that the JNK3 deficiency may reduce the severity of disease and may contribute to marked reduction in early mortality compared with SMA mice (Fig. 8B). These data suggest that the JNK3 deficiency improves the growth and survival of mice with SMA.

Further, to determine whether improvement in the SMA phenotype is because of reduced neurodegeneration or neuroprotection caused by JNK3 deficiency, we examined motor neurons in the spinal cords of Normal, SMA and SMA-J3 mice. Immunohistochemical staining for Choline Acetyltransferase (ChAT) and histochemical staining (H & E) of spinal cord sections show reduced loss of spinal motor neurons in SMA-J3 mice compared with SMA mice (Fig. 8C). Immunohistochemical staining with antibody to activated (cleaved) caspase 3 shows reduced cell death in spinal cord from SMA-J3 mice compared with SMA mice and suggests decrease in neurodegeneration (Fig. 8C, bottom panel). Comparison of number of motor neurons in the spinal cords (20 sections) of Normal (1462 ± 148), SMA (730 ± 124) and SMA-J3 (1197 ± 101) (mean ± s.d., three mice/group) mice shows that the JNK3 deficiency reduced loss of motor neurons to ~20% in SMA-J3 compared with ~50% loss in SMA mice, and provided neuroprotection by increasing survival of neurons ($81.92 \pm 3.99\%$, $P = 0.012$, t-test) in SMA-J3 mice compared with SMA ($49.95 \pm 4.90\%$) mice (Fig. 8D). These findings are consistent with the *in vitro* data that the inhibition of JNK3 activity results in reduced degeneration of SMN-deficient neurons (Fig. 6D).

The degeneration of spinal motor neurons leads to progressive muscle atrophy (muscle wasting) in SMA. It is possible that the reduced loss of motor neurons may improve muscle mass and account for increased growth in SMA-J3 mice. Examination of hind-limb muscle indicates increased muscle mass in SMA-J3 mice that is consistent with 1.75-fold ($P = 0.0023$) increase in body weight (Fig. 9A, top panel). Examination of gastrocnemius skeletal muscle longitudinal and transverse sections shows reduced muscle atrophy in SMA-J3 mice compared with SMA mice (Fig. 9A, middle and bottom panels). In addition, SMA-J3 mice also show reduction in connective tissue and increase in muscle fibers compared with SMA mice (Fig. 9A, bottom panel and 9B, top panel). Further examination of the muscle integrity and degeneration in skeletal muscle stained with antibodies to dystrophin and β -actin (markers for muscle morphology) indicate reduced muscle degeneration in transverse (H & E and Dystrophin) and longitudinal (Actin) gastrocnemius muscle sections of SMA-J3 compared with SMA mice (Fig. 9B). Analysis of muscle fiber diameter using a scatter plots shows reduced variation in myofiber size and increased mean diameter (μm) in SMA-J3 mice (18.38 ± 0.33) compared with SMA mice (13.39 ± 0.43) with $P < 0.0001$. Decrease in the negative slope of trend-line for SMA-J3 mice compared with SMA mice suggests reduced variation in myofiber size (Fig. 9C). Comparison of muscle fiber thickness

Figure 8. The *Jnk3* gene knockout in SMA mice reduces degeneration of spinal motor neurons, improves overall growth and increases lifespan of mice with SMA. (A) JNK3 deficiency improves the growth of mice with SMA. Growth (weight) curves of Normal [blue triangles], SMA-J3 [green squares] and SMA [red circles] mice littermates[†]. The results are presented as the mean $\pm$ s.e.m., $n = 5$ mice/group. Bar graphs show increase in number of growth days and increase in average peak weight of SMA-J3 mice compared with SMA mice. (B) Deletion of *Jnk3* gene increases lifespan of mice with SMA. Kaplan-Meier survival analysis of Normal (non-SMA) [blue], SMA-J3 [green] and SMA [red] mice. Bar graphs show increase in initial and average survival of SMA-J3 mice compared with SMA mice. Arrow indicates initial survival and arrowhead indicate maximum survival. (C) JNK3 deficiency reduces degeneration of spinal cord neurons in mice with SMA. Histochemical staining with hematoxylin and eosin (H & E) and immunohistochemical staining of the spinal cords sections from 8-day old Normal, SMA and SMA-J3 littermates[†] with anti-ChAT and cleaved caspase 3 (activated*) antibodies. Insets show ventral horn neurons. Arrows indicate degenerating neurons. Scale bar is 200 μ m, top and bottom panels, and 50 μ m, middle panel. (D) JNK3 deficiency provides neuroprotection and decreases the loss of spinal cord motor neurons. Bar Graph shows the loss of motor neurons in the thoracic (T9–T12) region of spinal cords from SMA and SMA-J3 mice compared with Normal (non-SMA) mice (littermates[†]) as the reference point (100%) (mean $\pm$ s.e.m.; three mice/group). [†]Littermates with all three genotypes present in the same litter.

(μ m) between Normal (22.73 ± 0.61), SMA (10.73 ± 0.31) and SMA-J3 (14.80 ± 0.24) show marked increase ($\sim 38\%$, $P = 0.0001$) in muscle fiber thickness in SMA-J3 mice (Fig. 9D).

To determine whether neuroprotection provided by JNK deficiency would help reduce muscle degeneration (muscle wasting), we examined gastrocnemius muscle areas lacking myofibers as indicated by arrows in Figure 9A, bottom panel and by asterisks in 9B, top panel using Nikon Imaging Software (NIS, Element (v4.2) as described in Supplementary Material, Fig. S4. Analysis of loss of muscle fiber (muscle wasting) by quantification of areas (μm^2) lacking muscle fibers (connective tissue) shows (24.60 ± 1.59)% loss in SMA and only (8.80 ± 1.15)% in SMA-J3. These data suggest that the JNK3 deficiency results in significant reduction ($\sim 16\%$, $P = 0.0041$) in muscle degeneration (wasting) of SMA-J3 mice compared with SMA mice (Fig. 9E). Furthermore, to test whether reduction in muscle degeneration improves muscle strength, we used hind-limb suspension test (HLST) to evaluate improvement in the proximal hind-limb muscle strength (33). Mice were hung on both hind legs on the edge of a 50 ml plastic conical tube and time was recorded until fall from edge of the tube (Fig. 9F). Comparison of latency to fall (seconds) between SMA-J3 (11.67 ± 1.45) and SMA (2.66 ± 0.67) shows marked (4.39-fold, $P = 0.0301$) increase in hanging time that suggests increase in hind-limb muscle strength of SMA-J3 mice. These data suggest

that the JNK3 deficiency reduces muscle degeneration, improves muscle growth and increases muscle strength of mice with SMA.

Defects in maturation of NMJs in SMA patients (34) and SMA mice are because of poor innervations caused by axonal retraction and neuron degeneration (6,35). To test whether neuroprotection provided by JNK deficiency will improve NMJs, we examined hind leg gastrocnemius skeletal muscle stained with antibody to neurofilament (NF-M, 145 kDa) protein and α -Bungarotoxin (BTX) to visualize nerve and acetylcholine receptors (AChRs), respectively. Comparison of innervated NMJs, indicated by colocalization (yellow) of NF (green) and AChRs (red), shows that the muscle from SMA-J3 mice contain higher number of innervated NMJs than SMA mice (Fig. 10A, upper panel). Examination of individual NMJs shows improved innervations of receptors in SMA-J3 mice compared with SMA mice that show NMJs with partial or full denervation (Fig. 10A, middle and lower panels). The denervation of NMJs due to neuron degeneration causes defects in synapse maintenance and may be responsible for NMJ synaptopathy in SMA (6,35). Synaptic defects, such as defect in synapse maturation and reduced synapse are shown to be present in animal models of SMA, including SMA $\Delta 7$ mouse model (8,36–38). To test whether JNK deficiency also improved synaptic maturation in SMA-J3 mice, we examined transversus abdominis (TVA) muscle stained with antibodies against synaptophysin and

Figure 9. JNK3 deficiency increases muscle growth, improves muscle fiber size and reduces muscle degeneration in mice with SMA. (A) JNK3 deficiency reduces muscle degeneration (muscle wasting) and increases muscle growth in mice with SMA. Histochemical staining (H & E) of hind limb (top panel), longitudinal sections (middle panel) and transverse sections of gastrocnemius muscle from 8-day old Normal, SMA and SMA-J3 littermates[†]. Arrows show connective tissue. Scale bar, 1 mm (top panel) and 50 μm (middle and bottom panels). (B) JNK3 deficiency reduces muscle degeneration and improves muscle fibers in mice with SMA. Histochemical staining (H & E) and immunohistochemical staining with antibodies to dystrophin (transverse) and β-actin (longitudinal) of gastrocnemius muscle from 8-day old Normal, SMA and SMA-J3 littermates[†]. Asterisks show areas of connective tissue. Scale bar is 50 μm. (C) JNK3 deficiency reduces variation in muscle fiber diameter of mice with SMA. The diameter of individual muscle fibers (microns) was calculated using transverse sections of gastrocnemius muscle from 8-day old Normal, SMA and SMA-J3 littermates[†] stained with hematoxylin and eosin (H & E). The scatter plots show distribution of myofiber diameters. Trend-line (black) slope show variation. The negative slope represents trend of decrease in diameter size. (D) The thickness of individual muscle fibers (microns) was calculated using longitudinal sections of gastrocnemius muscle from 8-day old Normal, SMA and SMA-J3 littermates[†] stained with β-actin. Thickness of muscle fibers (mean ± s.e.m., $n = 6$ sections/mice, three mice/group) plotted as a bar graph. Comparison of fiber thickness between SMA and SMA-J3 shows significant improvement (~38%, $P = 0.0001$, unpaired t-test) in muscle fiber. (E) Analysis of loss of muscle fiber (muscle wasting) by quantification of areas (μm²) lacking muscle fibers and connective tissue [as indicated by arrows in A, bottom panel] shown as bar graph (mean ± s.e.m., six sections/mice, three mice/group). Quantitation of areas was performed using Nikon Imaging System Software. A representative set of images showing areas used for quantification is included in Supplementary Material, Figure S3. Statistical analysis shows significant reduction (~16%, $P = 0.0041$) in muscle loss by JNK3 deficiency in SMA-J3 compared with SMA mice littermates[†]. (F) JNK3 deficiency improves muscle strength in mice with SMA. The HLST to evaluate improvement in the proximal hind-limb muscle strength was performed. Littermates[†] were hung on both hind legs on the edge of a 50 ml plastic conical tube and time (seconds) was recorded until fall from the edge of tube. Latency to fall (mean ± s.e.m., five mice/group) plotted as bar graph. Significant increase ($P = 0.0301$) in hanging time for SMA-J3 shows increase in muscle strength of SMA-J3 mice compared with SMA mice. [†]Littermates with all three genotypes present in the same litter.

neurofilament. SMA mice show NMJs with reduced innervation, retracting nerve fibers and reduced synaptophysin staining compared with SMA-J3 mice (Fig. 10B). Increase in NF staining in SMA-J3 ($98.10 \pm 4.10\%$) compared with SMA ($64.82 \pm 6.51\%$) mice [$P < 0.0001$, one-way ANOVA] indicates improvement in the density of nerve fibers that correlates with reduced loss of spinal motor neurons by JNK3 deficiency (Fig. 10B). Increased innervation and synaptophysin staining in NMJs of SMA-J3 compared with SMA mice indicate improvement in the synapse formation in SMA-J3 mice (Fig. 10B). These data suggest that the

neuroprotection achieved by JNK deficiency helps improve the innervations and functionality of NMJs in skeletal muscles of mice with SMA.

Different phenotypes of altered or reduced innervations or differences in arborization of NMJs by nerve fibers such as bud-like accumulation or undetectable differences in innervation between normal and SMA are reported by different studies (6,8,35,36,39). In this study, the staining pattern of neurofilaments in NMJs in gastrocnemius and TVA muscles from 8-day old mice with SMA is different from previously published studies

Figure 10. JNK3 deficiency improves innervation and synapse formation at NMJs in skeletal muscles of mice with SMA. (A) Immunohistochemical staining of gastrocnemius skeletal muscle with antibody to neurofilament M protein (NF, green) and α -Bungarotoxin coupled with Alexa 594 (BTX, red) from 8-day old Normal, SMA and SMA-J3 littermates[†]. Scale bars, 50 μ m (upper panel) and 10 μ m (middle and lower panels). (B) Immunohistochemical staining TVA skeletal muscle with antibodies to synaptophysin (red) and neurofilament M protein (NF, green) from 8-day old Normal, SMA and SMA-J3 littermates[†]. Scale bars, 20 μ m (upper and middle panels) and 10 μ m (lower panel). Arrowheads indicate partially denervated NMJs. Arrow shows retracting or degenerating nerve fiber. Asterisk shows non-innervated (or fully denervated) NMJs. [†]Littermates with all three genotypes present in the same litter.

(such as bud-like accumulation) and it might be because of a different clone (3H11) of neurofilament antibody (NF-M, 145 kDa) compared with other NF antibody clones such as NN18 (NF-M, 160 kDa) or SMI 312 (NF-H) or SMI-31 (phospho-NF-H) antibodies in comparison to gastrocnemius, TVA/LAL, TA, paraspinal or diaphragm muscle from mice with varying ages in several studies (6,8,35,36,39). It is possible that the recognition of unique epitopes on NF protein by individual antibody clones results in distinct patterns of staining. Nevertheless, SMA mice show muscle atrophy, partial and full denervation of NMJs and reduced

synapse formation during post-symptomatic development that are consistent with SMA pathogenesis (3,20,38,40). Together, these findings suggest that JNK3 deficiency reduces loss of motor neurons, decreases muscle degeneration, increases muscle fiber size and muscle growth, increases overall growth, improves motor function and increases lifespan of mice with SMA that represents a systemic partial rescue of SMA phenotype by genetic inhibition of JNK3.

Discussion

SMA is the leading genetic neuromuscular disorder of infant mortality with an incidence of 1:6000–10 000 worldwide. The severity of disease is classified into four types [SMA I (severe), II, III, IV] based on the age of onset, motor function ability and copy number of the SMN2 gene (4,41). The loss of spinal motor neurons leading to muscle weakness is the primary pathogenesis observed in all forms of SMA. The molecular mechanisms that mediate neurodegeneration in SMA are unclear. We show that the JNK pathway mediates neurodegeneration in SMA. We identified neuron-specific JNK isoforms, JNK3 as a potential target that mediates degeneration of SMN-deficient neurons. Genetic elimination of JNK3 reduces severity of SMA disease and results in partial but systemic rescue of phenotype in mice with SMA. We propose that the JNK signaling pathway mediates neurodegeneration in SMA and JNK3 represents a potential (non-SMN) therapeutic target to reduce the burden of illness in SMA.

Activation of the JNK signaling cascade in SMA

The role of JNK has been implicated in neurodegeneration (42). However, the intracellular mechanisms involved in JNK activation under pathological conditions of neurodegenerative disorders are unclear. Major challenges to unequivocally identify molecular events triggered by disease pathogenesis from deceased human subject samples include the rare possibility of multiple numbers of age-matched (2–6 months) controls (Normal, non-SMA), specific tissue such as spinal cord with specific region (lumbar region) and the PMI. For example, in one of the controls (non-SMA) sample (#82), male, age 137 days, post-mortem interval (37 h), cause of death complications of prematurity, we found that the tissue and proteins were severely degraded and this sample could not be used for analysis. Increase in phosphorylation of several kinases by MAPK array in SMA patient tissues suggests some non-specific activation that might be because of longer PMI. Comparison of data between SMA4583 and SMA4629 shows similar activity and that may be because of similar PMI of 4 and 3 h, respectively or similar disease severity. The sample SMA4994 shows higher activity that could be because of higher PMI (19 h) or increased severity. Importantly, Control (#83) that has highest postmortem interval (27 h) showed least activity compared with all SMA samples (Fig. 2). Statistical analysis and comparison of MAPK array data for common kinases activated in both human and mice helped eliminate non-specific activation; and suggested that the increase in JNK activity might be specific to SMA pathogenesis, and provided grounds for further testing and validation of JNK activation in SMA.

SMA is a developmental neuromuscular disorder with neuron degeneration during early postnatal development that results in early onset and stable, non-progressive course with gradual (chronic) increase in muscle weakness. It is possible that increase in age of patients and/or difference in severity of disease may contribute to higher kinase activity. Comparison of SMA patients with significantly different ages 77 days (SMA4583) and 169 days

(SMA4629) show similar amount of JNK activation in spinal cords, and these findings are consistent with data from SMA mice that show about 2-fold increase in activated JNK between the ages of 2-, 6- and 12-days (Fig. 3B). These findings suggest that aging may not cause a large increase in JNK activation during chronic SMA disease progression; however, difference in severity may affect levels of JNK activation as noted in SMA patients (SMA4583 and SMA4994).

The low levels of SMN in neurons may result in intracellular stress that might be a cause of activation of intracellular MAPK cascade, leading to JNK-mediated neurodegeneration in SMA. JNK is known to be activated by a variety of extracellular stress signals, including growth factors, cytokines and UV (26). However, mechanisms of JNK activation by intracellular stresses as a result of disease pathogenesis are relatively under studied. Activation of two MAP3Ks (ASK1 and MEKK1) and two MAP2Ks (MKK4 and MKK7) suggests higher order of regulation and specificity for JNK activation in SMA. The marked (12-fold) increase in levels of p-MKK4 compared with p-MKK7 indicates that MKK4 might be preferentially activated; however, small activation of MKK7 may be required for full *in vivo* activation of JNK (43). Activation of ASK1 and MEKK1 suggest the possibility of two signaling modules involved in the JNK activation because both MKK4 and MKK7 are known to be activated by ASK1 and MEKK1 (25). It has been reported that Gemin5, a part of the SMN complex, interact with ASK1, MKK4 and JNK in 293T cells (44). It is possible that Gemin5 might be a scaffold for ASK1/MKK4/JNK signaling module in neurons. Low levels of SMN complexes would result in a free pool of Gemin5 that might increase levels of ASK1/MKK4/JNK module in neurons. MEKK1 may contribute to MKK7 activation by another module MEKK1/MKK7/JNK scaffold by neuron-specific JNK-interacting protein3 (JIP3) (45). Together, these findings suggest that an increase in the levels of ASK1/MKK4/JNK module may be because of low levels of SMN associated with SMA pathogenesis. A graphical model of the activation of two JNK signaling modules in SMA is presented in Figure 11. Inhibition of the ERK pathway is shown to up regulate SMN2

expression that is mediated by activation of the AKT/CREB pathway in mice with severe SMA [*Smn*^{-/-}; *SMN2*^{+/+}] (23). Inhibition of the JNK pathway by deletion of *Jnk3* did not alter the levels of SMN in the spinal cord, brain or muscle tissues of mice with SMA. The decrease in AKT phosphorylation in SMA is consistent with suppression of AKT pathway during JNK activation (21). These findings suggest that JNK3 may not be involved in the regulation of SMN2 expression. However, the role of the JNK pathway in the regulation of SMN1 expression that generates several alternatively spliced transcripts (46), including axonal-specific SMN (a-SMN) isoform (47) that may contribute to SMA pathogenesis, remains to be studied.

Rescue and potential therapeutic targets of SMA

The lack of knowledge of the molecular mechanisms that mediate degeneration of motor neurons has hampered development of therapeutics, and no treatment is available to prevent or reduce neurodegeneration in SMA. Major efforts to develop SMA therapeutics are focused on increasing levels of SMN protein from the SMN2 gene by correcting splicing defects or enhancing transcription using multiple approaches (48), including antisense oligonucleotides (ASO) (49) and small cell permeable compounds such as histone deacetylase (HDAC) inhibitors (valproic acid, hydroxyl urea, trichostatin A, suberoylanilidehydroxamic acid and LDN-76070) (50,51), RNA decapping enzyme (DcpS) inhibitors (quinazoline compounds, RG3039) (52), activator of JAK2/STAT5 (prolactin) (53) and inhibitor of ERK/ELK-1 (U0126) (23) signaling pathways (48,54). One important question that needs to be addressed is whether restoring levels of SMN in degenerating neurons that have reached to a 'no point of return' will allow rescue of neurons to reintegrate and function normally. To address this, a strategy of combination therapy may be required that could include simultaneous treatments to (a) prevent or reduce degeneration of neurons (e.g. JNK inhibitors) and (b) increase levels of SMN using ASO-based approach or treatment with small cell permeable therapeutic compounds. Identification of non-SMN or SMN-independent drug targets is required to prevent neurodegeneration and development of successful treatments for SMA. Some progress has been made in identifying non-SMN targets, including (i) Plastin-3 (PLS3) (30), and ZPR1 (27) as modifiers of SMA, and (ii) RhoA/ROCK pathway (17). Overexpression of PLS3 in mice with SMA improves NMJ functionality by delaying axon pruning and moderately improves survival of mice (55). Inhibition of ROCK using inhibitors (Y-27632 and Fasudil) resulted in a partial rescue with improved NMJ functionality in skeletal muscle, and increase in survival of mice with intermediate SMA without affecting SMN levels (17,56). These findings indicate possibility of rescue of SMA phenotype by mechanisms independent of SMN (57). The genetic inhibition of *Jnk3* in mice with SMA reduced degeneration of motor neurons without affecting the levels of SMN in the brain, spinal cord and muscle tissues, suggests that the JNK pathway is independent of SMN and JNK3 represents a non-SMN target.

Degeneration of the spinal cord motor neurons is the primary cause of pathogenesis that leads to progressive muscle atrophy, respiratory failure and death in SMA. Therefore, inhibition of neurodegeneration in SMA may help reduce severity and rescue SMA phenotype. Deletion of the *Jnk3* gene in mice with SMA (SMA-J3) provided protection against neurodegeneration, improved NMJ innervations and functionality, improved muscle growth and motor function that resulted in partial systemic rescue of SMA phenotype and increased lifespan of mice with SMA. The reasons for a partial rescue are unclear. However, it is

Figure 11. Graphical representation of activation of the JNK signaling pathway in SMA. Two MAPK signaling modules (MAP3K → MAP2K → MAPK) (A) ASK1 → MKK4 → JNK3 and (B) MEKK1 → MKK7 → JNK3 found to be activated in the spinal cords of SMA mice and patients. *In vitro* elimination of JNK3 reduces degeneration of SMN-deficient neurons and provides neuroprotection. *In vivo* deletion of *Jnk3* in SMA mice results in partial systemic rescue and improvement in SMA phenotype.

possible that the partial rescue is because of (i) partial protection of neurons that might be due to functional redundancy of JNK isoforms because JNK1 can complement for JNK3 and mediate neurodegeneration (58); (ii) JNK has been shown to be activated in skeletal muscle cells (59) and cultured primary muscle from SMA patients (60). Therefore, JNK may also play a role in muscle wasting and other JNK isoforms, JNK1 and JNK2, might contribute to muscle degeneration in SMA, but remains to be studied; (iii) JNK3 deficiency may not be able to complement for splicing defects caused by SMN deficiency in neurons and skeletal muscle cells that result in reduced expression of (a) α -Nrxn2 in cultured neurons from severe SMA mouse model that might alter Ca^{2+} levels (61), (b) mature ryanodine receptor 1 that mediate calcium release from sarcoplasmic reticulum in SMA mice (62) and (c) sarcoplasmic reticulum Ca^{2+} ATPase [SERCA1a isoform predominantly express in TA skeletal muscle (63)] pump that remove Ca^{2+} from sarcoplasm in SMA mice (62) and (iv) since JNK3 predominantly express in CNS, the function of other non-neuronal tissues/organs such as heart (64–66), pancreas (67), liver (68,69), lung (70), vasculature (71–73), gastrointestinal system (74) and diaphragm/phrenic nerve (27) that regulate respiration (failure of respiration is the cause of death in SMA) (3) that are affected in SMA might not be improved by JNK3 deficiency. In addition to CNS, JNK3 has shown to be expressed in the heart, it is possible that the JNK3 deficiency might improve heart function, but remains to be tested (75).

Genetic inhibition of JNK3 reduces severity of disease, increases lifespan and ameliorates SMA phenotype. However, a raw comparison of survival of SMN Δ 7 mice with other published studies shows reduction in the average survival (~8 days), compared with average survival (11–13 days) reported in different studies with SMN Δ 7 mouse model (27,50,76). The original SMN Δ 7 mice line (Stock #005025) from the Jackson Lab shows survival of 7.6 ± 0.67 days, $n = 20$) under our laboratory conditions. These mice were crossed with *Jnk3*^{-/-} mice to generate SMA mice with *Jnk3* mutation (SMA-J3) and the survival of SMA pups (7.14 ± 0.93 days, $n = 21$) from SMA-J3 strain is similar to the survival of original SMA line. The decrease in average survival of original SMA Jackson strain might be because of several factors that could affect phenotype (survival) of SMA strain in different research laboratories, including diet, litter size, local environment, epigenetic modifications and any additional genetic modifications of the parent strain. It is established that the changes in the genetic content of SMA mouse models causes alteration in phenotype, including survival (77). To eliminate possibility of genetic variation, we analyzed and compared data from littermates produced by breeding of SMA-J3 carrier strain [*Smn*^{+/-}; *SMN2*^{+/-}; *SMN Δ 7*^{+/-}; *Jnk3*^{+/-}]. Therefore, the marked increase in the initial (4.20-fold), average (2.03-fold) and max survival by 7-days of SMA-J3 mice compared with SMN Δ 7 mice suggests that the JNK3 deficiency reduces severity of disease and increases survival of mice with SMA.

One of the important aspects of an ideal rescue of phenotype is whether increased survival is accompanied with reduced severity of disease. JNK3 deficiency resulted in 2.62-fold increase in embryonic growth of SMA-J3 mice compared with SMA mice indicating beneficial effects of JNK3 inhibition during embryonic development and suggesting a reduction in the severity of SMA. The increase in postnatal growth-period of SMA-J3 mice suggests decrease in postnatal severity of disease by elimination of JNK3. Increase in muscle fiber size and decrease in muscle wasting show improved muscle strength and gross motor function in SMA-J3 mice compared with SMA mice that supports reduction in severity of disease. Together, these findings suggest that

JNK3 deficiency prevented neurodegeneration and reduced the severity of disease from embryonic to postnatal stages that may account for marked 4-fold increase in initial survival, and may represent an important advancement towards developing alternative (SMN-independent) treatments to ameliorate SMA phenotype.

The role of JNK has been established in neuronal apoptosis and the JNK pathway has been indicated as a potential target for treatment of neurodegeneration in Alzheimer's and Parkinson's diseases (78). Our data suggest that the JNK signaling has potential for therapeutic intervention, and JNK3 may be a viable target to prevent neurodegeneration in SMA. A specific JNK3 inhibitor may be very useful in preventing neurodegeneration; however, functional redundancy of JNK isoforms may complement for JNK3 inhibition, but warrants further studies. It is possible that inhibitors with broad specificity (inhibits all JNK isoforms) may also be very useful in reducing neurodegeneration to rescue SMA phenotype (79). Alternatively, the inhibition of JNK signaling could also be achieved by targeting upstream kinases such as MKK4 and MKK7 (58). However, it remains to be tested whether pharmacological inhibition of JNK using JNK3 specific or broad specificity inhibitors will be effective in SMA mouse models. Nonetheless, improvement in SMA phenotype by genetic inhibition of JNK3 opens a gateway for exploring new avenues towards developing therapeutic interventions.

In conclusion, this study provides insight into the role of JNK3 (non-SMN target) in the pathogenesis and systemic improvement of SMA phenotype that will have important implications for developing alternative (SMN-independent) strategies for the treatment of SMA.

Materials and Methods

Mice

The wild-type (C57BL/6J) and *Jnk3*^{-/-} mice (19) were used to generate 7-day old pups. The SMA carrier mice on FVB background, [*Smn*^{+/-}; *SMN2*^{+/-}; *SMN Δ 7*^{+/-}] (20), purchased from the Jackson Laboratory were bred to generate non-SMA (normal) [*Smn*^{+/-}; *SMN2*^{+/-}; *SMN Δ 7*^{+/-}] and SMA [*Smn*^{-/-}; *SMN2*^{+/-}; *SMN Δ 7*^{+/-}] littermates. The *Jnk3*^{-/-} mice (C57BL/6 background received from Dr Roger Davis at the University of Massachusetts Medical School, Worcester) were backcrossed for 10 generations to wild-type FVB/N mice to create *Jnk3*^{-/-} (FVB background) mice. The SMA carrier mice [*Smn*^{+/-}; *SMN2*^{+/-}; *SMN Δ 7*^{+/-}] were crossed with *Jnk3*^{-/-} (FVB/N) mice to generate SMA mice with heterozygous *Jnk3*^{+/-}. SMA carrier mice with heterozygous *Jnk3*^{+/-} [*Smn*^{+/-}; *SMN2*^{+/-}; *SMN Δ 7*^{+/-}; *Jnk3*^{+/-}] were bred to generate mice with SMA-like disease [*Smn*^{-/-}; *SMN2*^{+/-}; *SMN Δ 7*^{+/-}; *Jnk3*^{+/-}] SMA (similar to SMA Δ 7) and [*Smn*^{-/-}; *SMN2*^{+/-}; *SMN Δ 7*^{+/-}; *Jnk3*^{-/-}] SMA-J3 (*Jnk3*-null background) littermates. The homozygous state of the two transgenes *SMN2*^{+/-} and *SMN Δ 7*^{+/-} was confirmed by breeding newly generated carrier mice (SMA-J3) breeder with wild-type mice, and litters were examined by PCR (20) for presence of SMN2 and SMN Δ 7 in all the pups (data not shown). Analyses of phenotypes were blinded and genotyping of littermates were performed after collection of data by PCR using tail DNA. Any combination of two or three pups with genotypes Normal, SMA [*Smn*^{-/-}; *SMN2*^{+/-}; *SMN Δ 7*^{+/-}; *Jnk3*^{+/-}] and SMA-J3 [*Smn*^{-/-}; *SMN2*^{+/-}; *SMN Δ 7*^{+/-}; *Jnk3*^{-/-}] in a litter was considered littermates. Mice were sacrificed at specific time points to collect tissues for biochemical and histological analysis with average postmortem interval less than 30 minutes. Animal studies were approved by the Institutional Animal Care and Use Committee (IACUC), TTUHSC.

SMA patient tissues

Human frozen spinal cord and brain tissues of (i) Control (non-SMA) sample-82, male, age 137 days, postmortem interval (37 h), cause of death complications of prematurity (born at 24 weeks of gestation), (ii) Control (non-SMA)-83, Age 69 days, Male, postmortem interval 27 h (cause of death Asphyxia), (iii) SMA type I-4994, Age 59 days, Female, postmortem interval 19 h (cause of death, complications of the disorder), (iv) SMA type I-4583, Age 77 days, Male, postmortem interval 4 h (cause of death Pneumonia) and (v) SMA type I-4629, Age 169 days, Male, postmortem interval 3 h (cause of death Cardiopulmonary Arrest) were received from the NICHD Brain and Tissue Bank for Developmental Disorders, Baltimore, MD 21201, USA. The Institutional Review Board (IRB) of the TTUHSC approved the use of human tissues.

MAPK array analysis

Standard and custom designed phospho-MAPK antibody arrays from the R&D Systems and Full-Moon BioSystems were processed according to manufacturer's protocol. Protein extracts were prepared from three individual spinal cords from mice (12-day old) or SMA patients either pooled or individually examined according to the manufacturer's protocols. Array images were analyzed using GenePix Pro software. Relative signal intensities normalized to β -actin or GAPDH (mean $\pm$ sem) were represented as bar graphs. One of the major differences between human and mouse tissue samples was the PMI. Average PMI for mouse was 30 min in contrast to 8.6 h for human. It is plausible that certain differences in data could be due to longer PMI of human tissues. We compared data for SMA mice and SMA patients and focused our analysis on kinases that were commonly activated in both SMA mice and SMA patients using two different MAPK arrays. The likely molecular targets were tested and confirmed by biochemical methods using mouse spinal cord tissues and cultured primary neurons. To reduce biological variation, protein extracts of spinal cords of three SMA patients or SMA mice were pooled and examined by phospho-MAPK antibody arrays.

Primary neuron culture and RNAi

Mouse spinal cord explants from 7-day old normal and SMA mice (littermates) were cultured *in vitro* for 10–12 days in 8-well chambers microscope slides, coated with poly-D-lysine/laminin using neurobasal medium supplemented with B-27, 700 mM Glucose, Glutamine, 2.5 mM KCl and penicillin/streptomycin with some modifications as described previously (27). Half of the culture medium was replaced with freshly made neurobasal medium every 48 h. The identity and morphology of the spinal cord motor neurons and glial cells were established by staining with specific markers, including ChAT and Hoxb9 (27). Primary CGNs were isolated from the cerebellum of 7-day old normal and SMA mice (littermates), WT and *Jnk3*^{-/-} mice and cultured *in vitro* for 6 days in 35 mm dish or 8-well chambers, coated with poly-D-lysine/laminin in neurobasal medium supplemented with B-27, glutamine, glucose and 2.5 mM KCl and penicillin/streptomycin (31). Neurons were transfected with 100 nM SMART pool siRNA (Dharmacon) specific for mouse *Smn* (M-044280-00) or Scramble II (Control, 5'-GCCGCTTTGTAGGATTCG-3') oligos and with pcDNA3/GFP-JNK3 (1 μ g) using Lipofectamine 2000 (80). Neurons were harvested 72 h post transfection and examined by IB and immunofluorescence (IF) analysis.

Immunoblot analysis

Human SMA patient type-I and non-SMA (Normal) age-matched frozen tissues (spinal cord and brain) were used for analysis. Tissues from different patients were pooled for extraction to reduce biological variation. Mouse spinal cord tissues were isolated from 12-day old non-SMA (Normal) and with SMA-like disease (SMA) littermates. Two spinal cords for each Normal, SMA set-1 (SMA-S1), set-2 (SMA-S2), set-3 (SMA-S3), set-4 (SMA-S4) were pooled from littermates for protein extraction. Protein extracts were prepared from cultured neurons, mouse tissues and human SMA patient tissues (brain and spinal cord) using Triton lysis buffer (81). Proteins were separated by SDS-PAGE and electrotransferred to PVDF membrane (Millipore). Immunoblot analysis was performed using primary antibodies (1:1000) followed by HRP-conjugated donkey anti-mouse or rabbit IgG (1:5000) secondary antibodies. The following primary antibodies specific for phospho and non-phospho proteins were used for analysis: Phospho-ASK1 (SAB4504337) and Phospho-MEKK1 (MAP3K1) #SAB4504617 (Sigma), ASK1 (D11C9), Phospho-MKK7 (Ser271/Thr275) #4171, MKK7 #4172, Phospho-MLK3 (Thr277/Ser281) #2811, MLK3 #2817, Phospho-SEK1/MKK4 (Ser257/Thr261) #9156, SEK1/MKK4 #9152, Phospho-SAPK/JNK (Thr183/Tyr185) (81E11) #4668; SAPK/JNK (56G8) #9258, Phospho-c-Jun (Ser63) #9261, c-Jun (60A8) #9165 (Cell Signaling). The other antibodies used for analysis include SMN (Clone 8, Transduction Lab), MEKK1 #ab55653, GFAP (GA5) #36700 (Cell Signaling), Cleaved Caspase-3 (Asp175) #9661. Chemiluminescence and quantitation of IBs were performed using ImageQuant LAS4000. The relative amounts of proteins (mean $\pm$ sem) normalized to either non-phospho protein or tubulin are presented as bar graphs.

Immunofluorescence analysis

Cerebellar granule neurons were isolated, purified from the cerebellum of 7-day old WT and *Jnk3*^{-/-} mice and cultured *in vitro* for 6 days in 8-well chambers coated with poly-D-lysine/laminin (31). Neurons were fixed with 4% paraformaldehyde (PFA), washed with PBS, permeabilized with 0.1% Triton-X100, blocked with 3% BSA and incubated with antibodies (82). Double labeling was performed by sequential incubations (1 h) with (a) antibodies against phospho-JNK #V793B (Promega) or phospho-c-Jun (Ser 63) or ChAT #ab6168 (Abcam) or Hoxb9 #92606 (Abcam), (b) Alexa 488-conjugated anti-mouse or rabbit IgG secondary antibody, (c) anti- β -Tubulin class-III neuron-specific, (TUJ1, Covance) and (d) Alexa 546-conjugated anti-rabbit or mouse IgG secondary antibodies (81). The GFP-JNK3 fluorescence was detected on excitation with 488 nm Laser (32). Cover slips were mounted on slides using Vectashield with DAPI (Vector Lab) to stain DNA and IF was examined using confocal microscopy [Leica SP5-AOBS equipped with 405 nm (UV) laser].

Immunohistochemistry

Mouse tissues, spinal cords and skeletal muscle from Normal (non-SMA), mice with SMA-like disease (SMA) and SMA mice with *Jnk3*^{-/-} (SMA-J3) littermates 8–12 day old were isolated, fixed in 4% PFA at 4°C for overnight, washed with PBS and then soaked in 30% sucrose solution for 48 h. Tissues were processed and embedded in paraffin or in OCT for frozen sections. Thin serial sections (10 μ m) were cut from the thoracic region (T9–T12) of the spinal cords and stained with hematoxylin and eosin or processed for immunohistochemical staining. Motor neurons were counted in every fifth section (20 sections) of the thoracic (T9–T12) regions of the spinal cord (27,82). For immunohistochemistry

staining, sections were processed for antigen retrieval, briefly incubated in SDS (1%) solution for 5 min and another 5–10 min in 0.1 M sodium citrate buffer maintained at 95°C. The spinal cord sections were rinsed in PBST, blocked in 3% BSA with 0.1% Triton-X100 for an hour and then incubated overnight with primary antibodies diluted (1:100) in 3% BSA (see list of antibodies in Immunoblot Analysis Section). Sections were washed five times in PBST, incubated for an hour with Alexa 488-conjugated anti-mouse or rabbit IgG secondary antibody diluted to 1:400 in 3%BSA, washed 4× with PBST and mounted with DAPI containing Vectashield (Vector Lab). Muscle sections (16 µm) from hind limb were rinsed with PBS to remove OCT compound and then incubated in blocking buffer (3% BSA with 0.1% Triton-X 100) for 1 h followed by 24 h incubation with primary antibodies to dystrophin (#15277, Abcam), β -actin (Sigma) or neurofilament (NF-M, 145 kDa) protein (#MAB1621, Millipore) diluted to 1:100 in 3% BSA. Next day, sections were washed with PBST (5× for 10 min), incubated for 3 h with Alexa 488 conjugated goat anti-rabbit or mouse secondary antibody (diluted to 1:100 in 3% BSA), followed by washing with PBST (5× for 5 min). Double immunolabeling staining was performed on sections stained with anti-neurofilament protein. The sections were incubated with 3% BSA for half an hour, followed by incubation with Alexa 594 conjugated α -Bungarotoxin (Invitrogen) or anti-synaptophysin antibody (Abcam) (diluted to 1:100 in 3% BSA) for an additional 3 h and rinsed with PBST (5× for 5 min) and slides were mounted using Vectashield. Immunofluorescence was examined by confocal microscopy.

Statistical analysis

The quantitative data are presented as mean $\pm$ s.e.m. Statistical significance (*P*-values) of data was calculated by one-way analysis of variance (ANOVA) analysis using GraphPad Prism or online software available at <http://www.danielsoper.com/statcalc/calc43.aspx>. Kaplan–Meier survival analysis, Log-rank (Mantel–Cox) test, Unpaired *t*-test with Welch's correction (two tailed) and Wilcoxon signed rank test (two tailed) were performed using GraphPad Prism (version 5.0d). The statistical value *P* = 0.05 or less was considered significant.

Supplementary Material

Supplementary Material is available at HMG online.

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Conflict of Interest statement. A patent application on the use of JNK inhibitors for the treatment of SMA has been filed by L.G.

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