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Regulation of Survival Motor Neuron Protein by the Nuclear Factor-Kappa B Pathway in Mouse Spinal Cord Motoneurons

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Abstract Survival motor neuron (SMN) protein deficiency causes the genetic neuromuscular disorder spinal muscular atrophy (SMA), characterized by spinal cord motoneuron degeneration. Since SMN protein level is critical to disease onset and severity, analysis of the mechanisms involved in SMN stability is one of the central goals of SMA research. Here, we describe the role of several members of the NF-κB pathway in regulating SMN in motoneurons. NF-κB is one of the main regulators of motoneuron survival and pharmacological inhibition of NF-κB pathway activity also induces mouse survival motor neuron (Smn) protein decrease. Using a lentiviral-based shRNA approach to reduce the expression of several members of NF-κB pathway, we observed that IKK and RelA knockdown caused Smn reduction in mouse-cultured motoneurons whereas IKK or RelB knockdown did not. Moreover, isolated motoneurons obtained from the severe SMA mouse model showed reduced protein levels of several NF-κB members and RelA phosphorylation. We describe the alteration of NF-κB pathway in SMA cells. In the context of recent studies suggesting regulation of altered intracellular pathways as a future pharmacological treatment of SMA, we propose the NF-κB pathway as a candidate in this new therapeutic approach.

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Introduction

The NF-κB (nuclear factor–κB) intracellular pathway is involved in physiological processes of the nervous system such as regulation of apoptosis, neurite outgrowth and synaptic plasticity [1–4]. A ubiquitously expressed transcription factor system, NF-κB consists of homodimers and heterodimers of five structurally related proteins: RelA/p65, RelB, c-Rel, p50 and p52, of which the p50/RelA heterodimer is the most abundant [5, 6]. In the absence of stimuli, NF-κB homo- or heterodimers are present in the cytoplasm and form inactive complexes with their inhibitors, the IκB (Inhibitor κB) protein family. When the pathway is activated, phosphorylation of IκB induces p50/RelA release and translocation to the nucleus. IκB is phosphorylated by the kinases of the inhibitor (IκB kinases), IKKα and IKKβ [7]. RelA can be phosphorylated at several sites inducing conformational changes which impact RelA ubiquitination, stability and protein-protein interactions. Phosphorylated RelA in S536 has lower affinity for IκBα, which results in increased nuclear translocation and accumulation of p65 [8]. In the nervous system, NF-κB activation regulates genes and proteins involved in several neuronal processes, such as Bcl-x_L [9], BDNF [10], NCAM [11] and Cu/Zn-SOD [12]. The canonical NF-κB activation regulates neuronal and motoneuron (MN) cell survival [13, 14], and evidences are emerging about its role in neurodegenerative disorders and neuronal injury [6, 15]. Based on NF-κB functions, it has been suggested that pharmacological regulation of NF-κB activity could be beneficial in some of these diseases [16].

Spinal muscular atrophy (SMA) is a genetic neuromuscular disorder characterized by the degeneration of MNs in the

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anterior horn of the spinal cord, together with muscular atrophy and weakness. SMA results from survival motor neuron (SMN) protein deficiency. In humans, SMN is encoded by the *SMN* gene, which is present in multiple copies, one telomeric copy of *SMN1* and several centromeric copies of *SMN2*. SMA is caused by homozygous disruption of the *SMN1* gene by deletion, conversion, or mutation [17]. *SMN1* expresses a full-length transcript, and *SMN2* expresses primarily a truncated isoform that is unable to compensate *SMN1* deficiency. SMN protein level is critical to disease onset and severity and is determined in part by the *SMN2* copy numbers [18]. One of the central objectives of developing SMA therapeutics is to identify mechanisms that increase SMN protein levels [19], which require a basic understanding of SMN protein regulation. In the present study, we characterized the contribution of several members of the NF-κB pathway in regulating mouse survival motor neuron (Smn) protein level in cultured spinal cord MNs. Pharmacological inhibition of NF-κB pathway activity induced Smn protein decrease. Using a lentiviral-based short-hairpin RNA (shRNA) approach to reduce the expression of several members of the NF-κB pathway, we observed that IKKα and RelA knockdown caused Smn protein reduction whereas IKKβ or RelB knockdown did not. Isolated and cultured MNs from severe SMA mouse model showed reduced protein levels in several members of NF-κB and RelA phosphorylation. Our results suggest that NF-κB pathway activation regulates SMN protein level in spinal cord MNs.

Materials and Methods

Mouse MN Isolation and Culture

MN cultures were prepared from embryonic 12.5-day (E12.5) CD1 male and female mouse spinal cords essentially as described [20, 21]. Isolated cells were pooled in a tube containing culture medium and plated [21]. Cultured MNs enriched by Iodixanol were clearly identified by morphological criteria. All of the procedures were in accordance with the Spanish Council on Animal Care and approved by the University of Lleida Advisory Committee on Animal Services.

Isolated MNs were plated in four-well tissue culture dishes (Nunc, Thermo Fisher Scientific) in a density of 15,000 cells per well for survival and immunofluorescence experiments, 70,000 cells per well for western blot analysis and 300,000 cells per well for qRT-PCR. Complete culture medium was Neurobasal (Gibco, Invitrogen) supplemented with B27 (Gibco, Invitrogen), horse serum (2% *v*/*v*), L-glutamine (0.5 mM), and 2-mercaptoethanol (25 μM). Cells were plated and maintained with complete medium containing a cocktail of recombinant NTFs: 1 ng/ml brain-derived neurotrophic factor, 10 ng/ml glial cell line-derived neurotrophic factor, 10 ng/ml cardiotrophin-1, and 10 ng/ml hepatocyte growth factor (Peprotech).

Survival evaluation was performed using photomicrographs of different microscopic areas from each dish (4 central areas per well, 3 wells for each condition), and counting the number of large-phase bright neurons with long neurite processes present in the photomicrographs. Survival was expressed as the percentage of cells counted 3 days after treatment with respect to the initial value (3 h after plating, 100%).

SMA Animals

Severe SMA mice FVB.Cg-Tg(SMN2)^{89Ahmb}Smn1^{tm1Msd}/J were kindly provided by Dr. Josep E Esquerda (IRBLLEIDA-Universitat de Lleida). Heterozygous animals were crossed to obtain homozygous *Smn*$^{-/-}$;SMN2$^{+/+}$ (mutSMA). Littermates mutSMA and *Smn*$^{+/+}$;SMN2$^{+/+}$ (WT) were used for the experiments. For MN purification, E13 embryos were removed from the uterus and a piece was snipped from the head for genotyping. The REDExtract-N-Amp Tissue PCR Kit (Sigma) was used for genomic DNA extraction and polymerase chain reaction setup, with the following primers: WT forward 5′ CTCCGGATATTGGGATTG 3′, SMA reverse 5′ GGTAACGCCAGGGTTTTCC 3′ and WT reverse 5′TTTCTTCTGGCTGTGCCTTT 3′. After genotyping, WT and mutSMA embryos were submitted to spinal cord dissection, MN isolation and culture as described above.

Immunofluorescence of RelA Nuclear Translocation

The determination of cellular localization of RelA into MNs was performed as previously described [13]. Briefly, cultures were fixed 5 days after lentivirus transfection in 4% paraformaldehyde and 100% methanol, incubated overnight at 4 °C with a polyclonal anti-RelA antibody (1:100; Santa Cruz Biotechnology), and consecutively with an anti-rabbit secondary antibody conjugated with Alexa Fluor 488 (Invitrogen). Hoechst 33,258 staining was used for nuclear localization. The images were obtained using a FluoView 500 Olympus confocal microscope.

Western Blot Analysis

Western blot analysis was performed as previously described [22]. Cells were rinsed in ice-cold PBS (pH 7.2) after stimulation or lentiviral transduction. Total cell lysates were collected and resolved in SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride Immobilon-P transfer membrane filters (Millipore, Billerica, MA) using an Amersham Biosciences (Piscataway, NJ, USA) semidry Trans-Blot according to manufacturer instructions. Membranes were blotted with the specific antibodies anti-RelA (1:1000), anti-

phosphoRelA (Ser536) (1:1000) or anti-CREB (1:1000) from Cell Signaling Technology; anti-SMN (1:5000) from BD Transduction Laboratories; anti-IKKβ (1:1000) or anti-IKKα (1:1000) from Calbiochem or anti-RelB (1:1000) (C-19) from Santa Cruz, following the instructions of the providers. To control the specific protein content per lane, unless stated otherwise, the membranes were re-probed with a monoclonal anti-α-tubulin (Tub) antibody (1:50,000, Sigma), as described by the provider. Blots were developed using the Super Signal chemiluminescent substrate (Pierce, Rockford, IL, USA) or the ECL Advance Western Blotting detection kit (Amersham Biosciences).

NF-κB inhibitor SN50, the inactive control peptide SN50M, and the proteasome inhibitor MG132 were purchased from Calbiochem.

RNA Isolation and Quantitative RT-PCR

For qRT-PCR analysis, MNs (300,000 cells/condition) were plated and transduced with shRNA. Total RNA was extracted using the NucleoSpin® RNA columns (Machery-Nagel, Düren, Germany) according to manufacturer instructions. One microgram of total RNA from each condition was reverse-transcribed to complementary (cDNA), and 25 ng was used for each individual RT-PCR reaction. The assays were performed in a CFX96 Real-Time System (Bio-Rad) using iTaq™ Universal SYBR® Green Supermix from Bio-Rad. Real-Time was performed using SMN-specific primers: SMN exon 1-forward (5′-GATGATTCTGACATTTGGGATG-3′) and SMN Exon 2-reverse (5′-TGGCTTATCTGGAGTTTCAGAA-3′) and specific primers of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*): forward (5′-TGCACCACCAACTGCTTAG-3′) and reverse (5′-GGATGCAGGGATGATGTTC-3′) as internal control. Quantification was completed using iCycler IQ real-time detection system software (version 2.3, Bio-Rad). Relative expression ratios were calculated on the basis of ΔCp values with efficiency correction based on multiple samples.

Plasmids and Production of Lentiviral Particles

Lentiviral-based vectors for RNA interference-mediated gene silencing (FSVi) were performed as described [23]. FSVi consisted of a U6 promoter for expression of short-hairpin RNAs (shRNAs) and the Venus variant of green fluorescent protein (GFP) under the control of an SV40 promoter to monitor transduction efficiency. Lentiviruses were propagated in HEK293T cells using the polyethylenimine (PEI, Sigma) cell transfection method. Twenty micrograms of the above plasmids containing the shRNAs or the empty vector (FSV), 13 μg of pSPAX2 and 7 μg of pM2G were transfected to HEK293T cultures. Cells were allowed to produce lentivirus for 4 days. Then, the medium was centrifuged at 1200×*g* for 5 min and the supernatant was filtered using a 45 μm filter. The medium containing the lentiviruses was stored at 4 °C. Biological titres of the viral preparations, expressed as the number of transducing units per ml (TU/ml), were determined by transducing HEK293T cells in limiting dilutions. After 48 h, the percentage of GFP-positive cells was measured and viruses at 4×10^5–1×10^6 TU/ml were used for the experiments. For lentiviral transduction, MNs were plated in four-well dishes and after 2 h, the medium containing lentivirus (2 TU/cell) was added. The medium was changed 20 h later and infection efficiency was monitored in each experiment by direct-counting GFP-positive cells. The frequency of infection rose 99%. RNA interference efficiency was monitored by western blot analysis using specific antibodies.

The following sequences were used for generation of shRNAs: IKKα: 5′-CAGGCTCTTTCAGGGCAT-3′; IKKβ: 5′-GCTGCACATTTGAATCTGTAT-3′; RelA: 5′-AGATCTTGAGCTCGGCAGTG-3′; RelB: 5′-GCATGCGCTTCCGCTACGAGT-3′; CREB: 5′-GTGGATAGTGTAACTGATT-3′. shRNAs of IKKs and RelA have been previously described [24].

Statistical Analysis

All experiments were performed at least three times. Values were expressed as mean ± SEM. The data obtained from the independent experiments were used for statistical analysis.

We used one-way analysis of variance (ANOVA) to assess differences between groups for variable treatment. If the ANOVA test was statistically significant, we performed "post-hoc" pairwise comparisons using the Bonferroni test. We used the Student *t* test to compare the means of two groups. P values < 0.05 were considered significant.

Results

NF-κB Inhibitor SN50 Reduces Smn Protein Level in Cultured Spinal Cord MNs

Previous studies have shown that NF-κB pathway contributes to neurotrophic factor (NTF)-mediated MN survival [13]. To further investigate the role of NF-κB in MN physiology and Smn regulation, we pharmacologically inhibited the NF-κB pathway using the SN50 inhibitor peptide and analysed Smn levels in protein extracts of cultured MNs. SN50, a cell-permeable peptide that blocks the nuclear localization sequence of p50, inhibits the translocation of NF-κB active complex to the nucleus [25]. MNs were isolated from E12.5 mouse embryos and maintained in the presence of the NTF cocktail (1 ng/ml BDNF, 10 ng/ml GDNF, 10 ng/ml CNTF and 10 ng/ml HGF). Cultures were left untreated (control) or treated with 30 μM SN50 or its inactive control peptide,

SN50M. Two days later, cell extracts were collected and analysed by western blot using a specific antibody against Smn (Fig. 1). The presence of the NF-κB inhibitor in the culture medium significantly decreased Smn protein level, whereas its negative control SN50M did not induce significant changes in Smn (0.42 ± 0.1 SN50 vs control $p < 0.05$ and 0.86 ± 0.07 SN50M vs control). Survival evaluation after 2 days of treatment showed a significant decrease from baseline in the number of MNs in SN50-supplemented cultures, compared with SN50M (16.1 ± 1.3% SN50 and 82.3 ± 4.1% SN50M, $p < 0.0001$) and an increase of apoptotic nuclei (data not shown). These results indicate that the pharmacological inhibition of the NF-κB pathway reduces survival and Smn protein level in cultured mouse spinal cord MNs.

RelA, But Not RelB, Inhibition Causes Smn Reduction in MNs in the Presence of NTFs

To determine whether the canonical or non-canonical NF-κB pathways are relevant in Smn protein regulation in MNs, we analysed the effect of RelA or RelB knockdown. The RelA/p50 heterodimer is activated in the canonical and RelB/p52 in the non-canonical pathway [7]. Therefore, we used shRNA targeting specific sites of mouse RelA (shRelA) or RelB (shRelB) sequences to block the canonical or non-canonical pathways, respectively [13]. MNs were isolated from E12.5 mouse embryos and maintained in the presence of NTFs. Two hours after plating, culture medium was changed and medium containing NTFs plus lentivirus of shRelA or shRelB or shRNA empty vector (FSV) was added. Twenty hours later, the lentivirus-containing medium was washed and replaced by fresh medium supplemented with NTFs. Fluorescence microscopy of GFP-positive cells showed nearly 100% transduction (Fig. 2). After 5 days, western blot analysis of cultures transduced with the lentivirus carrying the shRelA exhibited a significant reduction in Smn protein (0.57 ± 0.03; $p < 0.05$) whereas cultures transduced with shRelB showed no change (0.99 ± 0.06) (Fig. 2a, b, respectively). Control western blots of RelA or RelB protein revealed significant reductions in both cases, under shRelA or shRelB conditions, respectively. These results showed NF-κB pathway regulating Smn protein level by the canonical pathway, but not by the non-canonical pathway.

Both IKKα and IKKβ Knockdown Prevent RelA Phosphorylation and Nuclear Translocation in Cultured MNs

RelA phosphorylation and nuclear translocation is a key event in canonical NF-κB activation and target gene regulation. To analyse the effect of IKKα or IKKβ reduction on RelA phosphorylation and translocation to the nucleus, MNs were transduced with shIKKα, shIKKβ or empty vector (FSV) or were

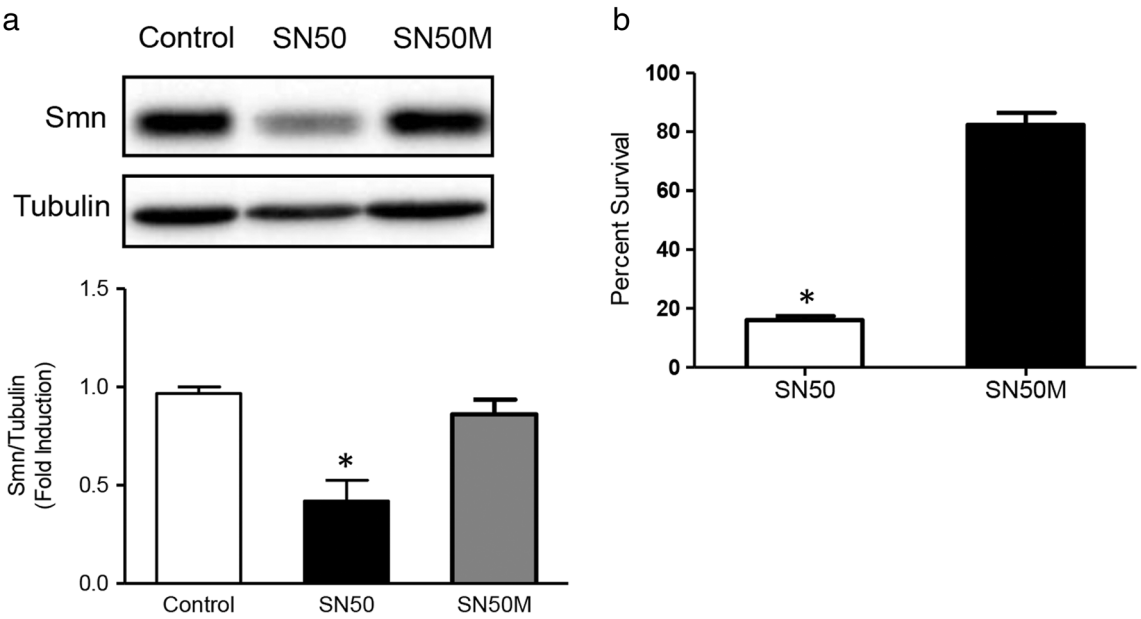

Fig. 1 Effect of NF-κB inhibitor SN50 on Smn protein level. **a** Representative Western blots for Smn and α-tubulin (loading control) in protein extracts of MNs treated during 2 days with the NF-κB inhibitor SN50 (30 μM) and its inactive control SN50M (30 μM). Graph values indicate the expression of Smn vs tubulin and correspond to the quantification of three independent experiments ± SEM (error bars). Asterisk indicates significant differences using one-way ANOVA test and Bonferroni post hoc multiple comparisons ($p < 0.05$). **b** MNs were cultured in the presence of SN50 (30 μM) or SN50M (30 μM). Cell survival was evaluated 2 days after treatment. Graph values are the mean of the percentage of cell survival for each condition from three independent experiments ± SEM. Asterisk indicates significant differences using Student *t* test ($p < 0.001$)

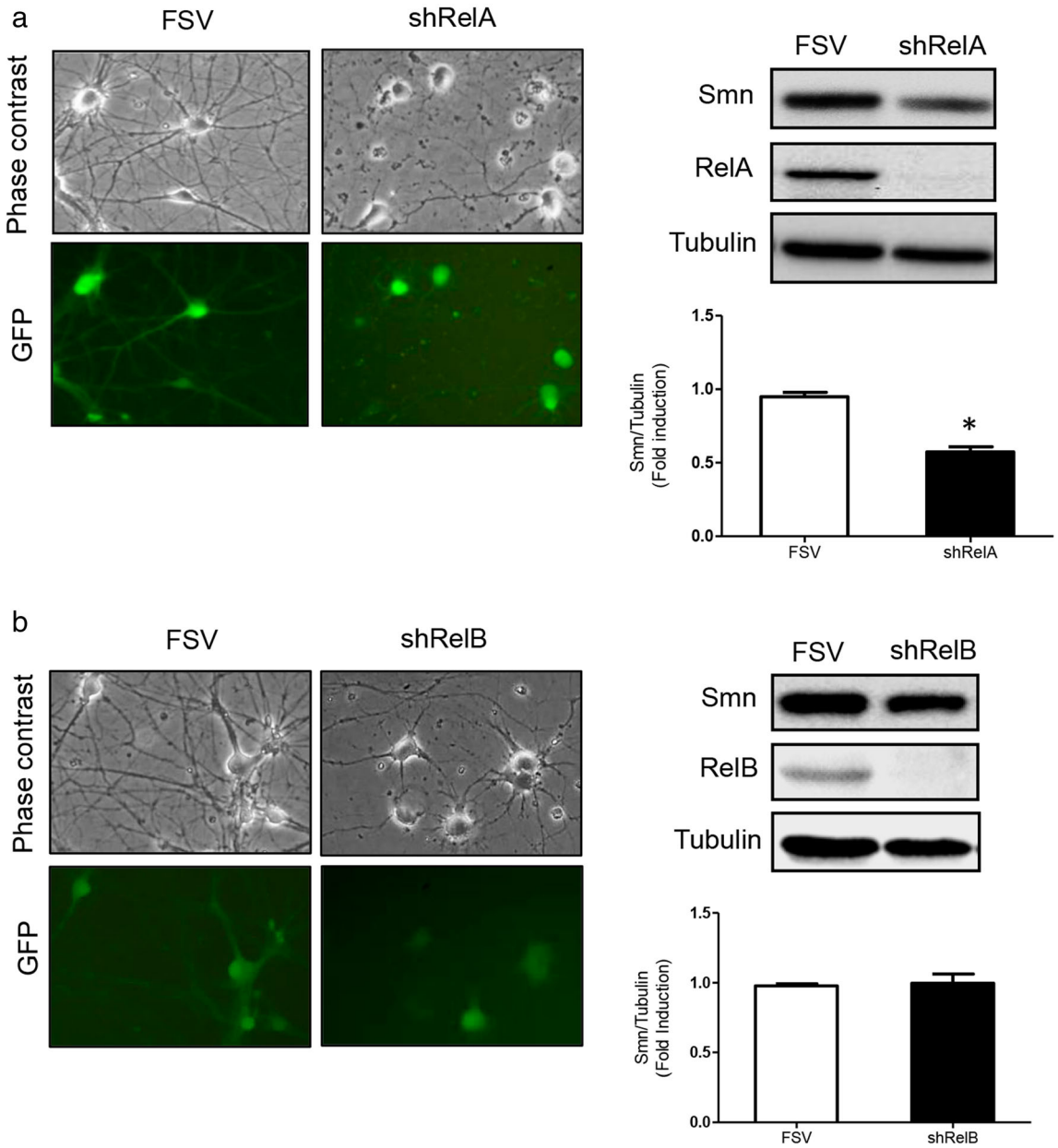

Fig. 2 RelA, but not RelB, knockdown reduced Smn protein level in cultured spinal cord MNs. Mouse MNs were transduced with lentivirus containing the shRelA, shRelB or empty vector (FSV) constructs. **a** Representative microscopy images of 5-day shRelA- or FSV-transduced cells: phase contrast (top) and GFP (bottom). Total cell lysates were probed by western blot with anti-SMN or anti-RelA antibodies and reprobed using an antibody against α-tubulin. Graph values represent the expression of Smn vs α-tubulin and correspond to the quantification of three independent experiments ± SEM. Asterisk indicates significant differences using Student *t* test ($p < 0.05$). **b** Representative microscopy images of 5 days shRelB- or FSV-transduced cells: phase contrast (top) and GFP (bottom). Total cell lysates were probed by western blot with anti-SMN or anti-RelB antibodies and reprobed using an antibody against α-tubulin. Graph values represent the expression of Smn vs α-tubulin and correspond to the quantification of three independent experiments ± SEM

left non-transduced (control). After 5 days, cultures were submitted to western blot analysis using anti-phosphoRelA (pRelA) or anti-RelA antibodies or were fixed to determine RelA localization by immunofluorescence using an anti-RelA antibody. RelA phosphorylation was significantly reduced in shIKKα (0.66 ± 0.02, $p < 0.001$) and shIKKβ (0.31 ± 0.05, $p < 0.0001$) cultures compared with the FSV control (Fig. 3a). It was also evident that pRelA level was significantly reduced in shIKKβ compared with shIKKα cultures ($p < 0.001$). However, no differences were observed in total RelA level, indicating that the reduction of RelA phosphorylation was not due to RelA protein decrease.

In control cultures, immunofluorescent confocal images of cultured MNs using an anti-RelA antibody showed RelA localization clearly distinguishable into the nucleus (Fig. 3b). Hoechst33258 was used as a nuclear marker. We measured the

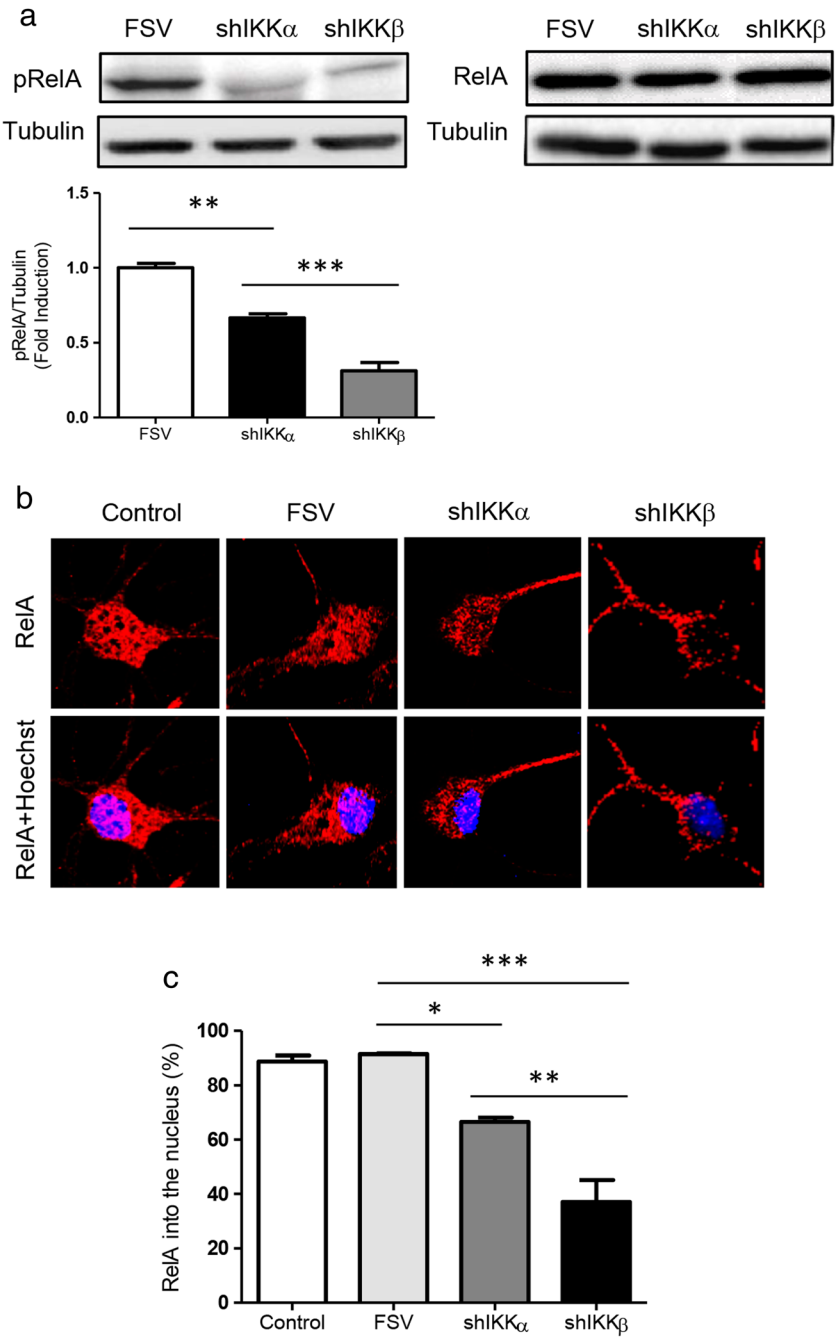

Fig. 3 Effect of IKK knockdown on RelA phosphorylation and nuclear translocation. Mouse MNs were transduced with lentivirus containing the shIKKα, shIKKβ or empty vector (FSV) constructs. **a** Representative western blot for phospho-Ser536 RelA (pRelA) and total RelA in protein extracts of 5-day transduced cultures. Membranes were reprobed using an antibody against α-tubulin. Graph values represent the expression of p-RelA or RelA vs α-tubulin and correspond to the quantification of three independent experiments ± SEM. **b** Five-day transduced cells were fixed and immunofluorescence was performed using an anti-RelA antibody. Representative confocal images of p65 (red) and Hoechst nuclear staining (blue). Graph represents the percentage of cells with nuclear RelA in each condition and corresponds to the quantification of three independent experiments ± SEM. In **a** and **b,** asterisks indicate significant differences using one-way ANOVA test and Bonferroni's post hoc multiple comparisons (***$p < 0.0001$, **$p < 0.001$, *$p < 0.01$)

percentage of MNs with nuclear RelA in control, FSV, shIKKα and shIKKβ cultures. Cellular distribution showed a reduced percentage of MNs with nuclear RelA in shIKKα (66.5 ± 1.6%) and shIKKβ (37.1 ± 8.1%) conditions, compared to FSV (91.5 ± 0.2%; $p < 0.01$ and $p < 0.0001$, respectively). No significant differences in nuclear RelA were observed in non-lentivirus treated (control) and FSV conditions (88.8 ± 2.2 and 91.5 ± 0.4%, respectively). These results also indicate a decreased percentage of nuclear RelA in IKKβ knockdown cells, compared with shIKKα cultures ($p < 0.001$), supporting the hypothesis that IKKβ is the main kinase responsible for RelA phosphorylation.

Inhibiting IKKα Vs IKKβ has Opposite Effect on Smn Protein Level in MNs

Because the activation of the canonical NF-κB pathway depends upon the phosphorylation status of the two kinases IKKα and IKKβ, we analysed the effect of IKK knockdown on the Smn level of cultured MNs. To this end, we produced lentivirus containing the shRNA targeting specific sites of mouse IKKα (shIKKα) or IKKβ (shIKKβ), as previously described [13]; MNs were isolated and transduced following the same protocol. After 5 days, cultures transduced with shIKKα or

shIKKβ exhibited a strong reduction in IKKα or IKKβ protein, respectively, compared with the empty vector condition (FSV). Western blot analysis of protein extracts of shIKKα condition showed a significant reduction in Smn protein (0.62 ± 0.07, $p < 0.001$) compared to the FSV or non-transduced (control) conditions (Fig. 4a). To discard changes in Smn after lentiviral transduction, protein extracts from non-transduced cultures were submitted to western blot; no differences were observed when compared with the FSV condition. When cells were transduced with the shIKKβ, Smn protein level was significantly increased (1.3 ± 0.13, $p < 0.005$) compared to the controls (Fig. 4a). These findings demonstrated

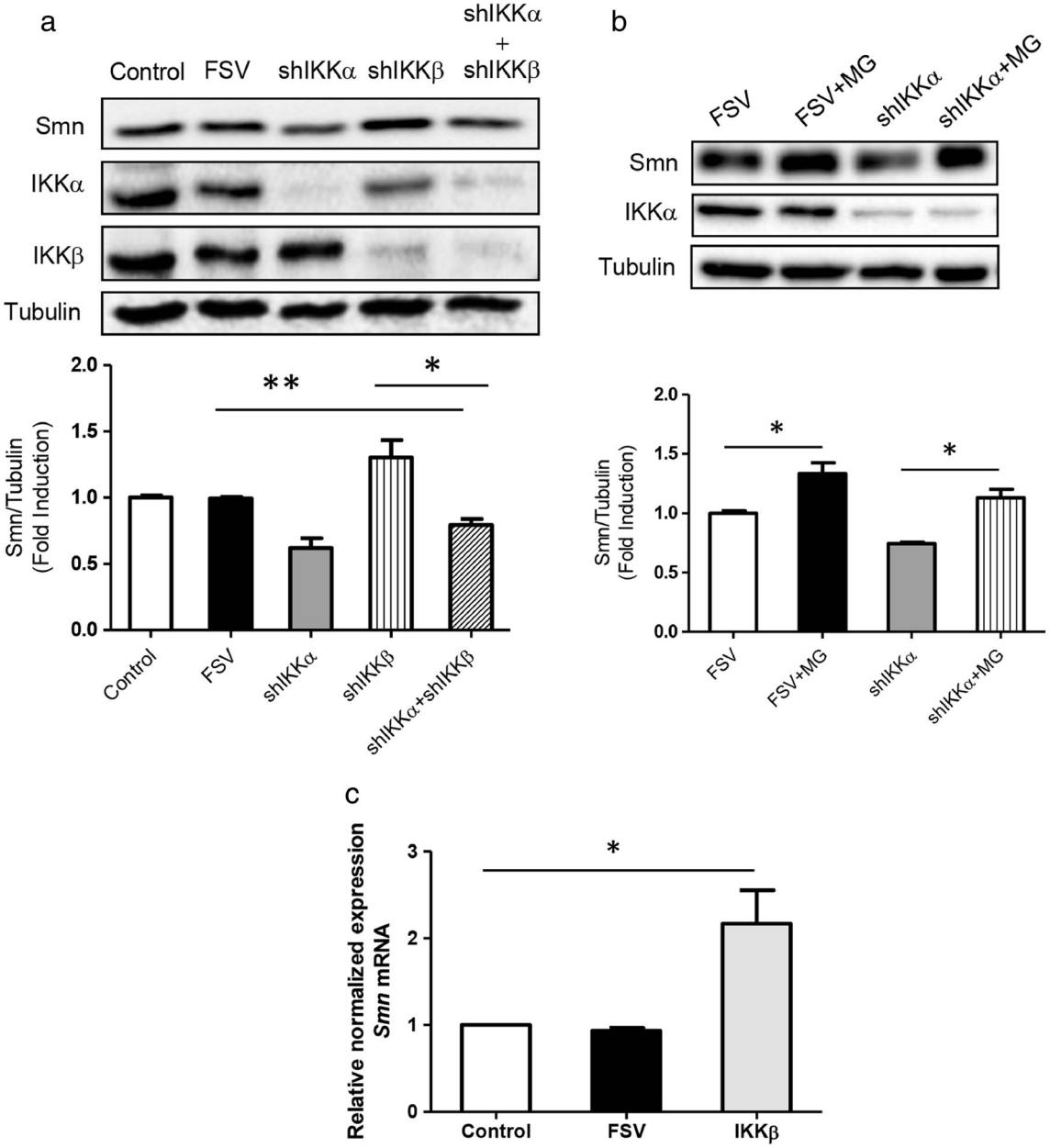

Fig. 4 Smn protein and mRNA regulation by IKK knockdown. MNs were transduced with lentivirus containing the shIKKα, shIKKβ, shIKKα + shIKKβ or empty vector (FSV) constructs. **a** Protein extracts of 5-day transduced cells were submitted to western blot using anti-SMN, anti-IKKα and anti-IKKβ antibodies. **b** Five-day FSV and shIKKα transduced cultures were treated (12 h) with 2.5 μM MG132. Protein extracts were submitted to western blot using anti-SMN and anti-IKKα antibodies. In **a** and **b**, membranes were reprobed with an anti-α-tubulin antibody. Graph values represent the expression of Smn vs α-tubulin and correspond to the quantification of 5 (**a**) or 3 (**b**) independent experiments ± SEM. Asterisks indicate significant differences using one-way ANOVA test and Bonferroni's post hoc multiple comparisons (*$p < 0.05$). **c** Total RNA was extracted from 5-day FSV-, shIKKα- and non-transduced cultures and reverse-transcribed to cDNA. *Gapdh* gene was used as a control. Graph values are the mean of *Smn* gene expression for each from three independent experiments ± SEM. Asterisks indicate significant differences using one-way ANOVA test and Bonferroni's post hoc multiple comparisons (*$p < 0.05$)

that both IKKα and IKKβ regulate Smn level in MNs. Nevertheless, IKKα reduction decreases Smn level whereas IKKβ knockdown increases Smn count.

Because IKKα and IKKβ knockdown produced opposite effects on Smn levels, we analysed the effect of shIKKα and shIKKβ co-transduction on Smn protein level. Five days after, shIKKα and shIKKβ co-transduction protein lysates were obtained and submitted to western blot analysis (Fig. 4a). Results revealed that Smn protein was not significantly altered in co-transduced cultures compared with FSV or non-transduced controls. However, Smn level in the co-transduced condition (0.82 ± 0.08) was significantly reduced ($p < 0.05$) in comparison with shIKKβ (1.03 ± 0.13). Therefore, IKKα knockdown clearly prevents Smn increase caused by IKKβ reduction.

To further analyse Smn regulation by IKKα knockdown, we evaluated the effect of proteasome inhibition. Previous studies have shown that SMN degradation is mediated via the ubiquitin/proteasome pathway in SMA patient-derived fibroblasts [26, 27]. Cells were transduced with FSV or shIKKα. Five days after transduction, cultures were treated for 12 h with 2.5 μM of the proteasome inhibitor MG132. Protein extracts were submitted to western blot using the anti-SMN antibody. Under both FSV and shIKKα conditions, Smn protein level was increased in MG132-treated cells compared with the non-treated FSV or shIKKα condition (1.33 ± 0.09-fold induction and 1.13 ± 0.07-fold induction, respectively, $p < 0.05$) (Fig. 4b). This result suggests the Smn decrease caused by IKKα knockdown occurs as a result of the proteasome activity.

Next, to determine whether the Smn increase caused by IKKβ knockdown was associated with activation of *Smn* gene expression, we quantified *Smn* messenger RNA (mRNA) by quantitative RT-PCR (qRT-PCR). *Gapdh* gene was used as a control. Cultured MNs were transduced with FSV or shIKKβ lentiviral constructs. After 4 days, total RNA was extracted and reverse-transcribed to cDNA, used as a template to quantify *Smn* transcript level. IKKβ reduction was related to an increase in *Smn* mRNA expression (2.17 ± 0.38-fold induction, $p < 0.05$) compared with control conditions (non-transduced and FSV), showing that IKKβ knockdown regulates Smn at the transcriptional level (Fig. 4c).

IKKα or IKKβ Knockdown Affects CREB Protein Levels in MNs

Previous evidence suggests that cAMP-response element-binding (CREB) may regulate *SMN* expression [28]. In turn, NF-κB signalling pathway controls CREB protein level in MNs [13, 28]. In this context, we decided to analyse CREB protein level in IKK knockdown cultures. MNs were isolated and transduced with empty vector (FSV) or shIKKα, shIKKβ or shIKKα plus shIKKβ. Twenty-four hours after transduction, medium containing lentiviral particles was replaced with fresh medium supplemented with NTFs. Five days later, cells were lysed and protein extracts were submitted to western blot analysis. The CREB protein level was significantly reduced in shIKKα-transduced MNs (0.66 ± 0.02, $p < 0.001$), compared to the FSV control. In contrast, IKKβ knockdown significantly increased the level of CREB protein (1.79 ± 0.1, $p < 0.005$), compared to FSV control. When cultures were co-transduced with shIKKα plus shIKKβ, the level of CREB protein (0.59 ± 0.09, $p < 0.05$) was comparable to that observed in shIKKα-only cultures (Fig. 5a). These results show that the IKK complex regulates the intracellular level of the CREB protein with a pattern similar to that observed in Smn experiments.

To analyse whether CREB reduction causes changes in Smn protein level, we generated lentivirus shCREB, isolated the MNs, and transduced the cells with the empty vector (FSV) or shCREB. After 24 h, the medium was replaced with NTF-supplemented medium and cells were maintained in this medium for 5 days. To measure the level of Smn protein, total protein extracts were submitted to western blot analysis. The Smn protein level was significantly reduced in CREB knockdown condition (0.82 ± 0.02, $p < 0.05$), compared with the FSV control (Fig. 5b). These experiments suggest that the CREB transcription factor regulates Smn protein level in MNs.

NF-κB Levels in Cultured MNs Differ between Wild-Type and SMA Mutant Mice

To analyse whether levels of NF-κB pathway proteins were altered in SMA-mutant MNs, E13 embryos of SMA type I mice were genotyped and spinal cords of WT ($Smn^{+/+}$;$SMN2^{+/+}$) and mutSMA ($Smn^{-/-}$;$SMN2^{+/+}$) were dissected. MNs were isolated following the same protocol described in the "Materials and Methods" section. Cells were maintained in the presence of NTFs for 6 days before cell lysates were obtained. Western blot analysis of total protein extracts demonstrated that IKKα and IKKβ were significantly reduced in mutSMA (0.85 ± 0.03, $p < 0.005$; 0.67 ± 0.06, $p < 0.005$, respectively) compared to the WT condition (Fig. 6a). A time-course experiment demonstrated that IKKα and IKKβ reduction was maintained from day 2 to day 8 in culture (data not shown). The level of RelA protein (0.92 ± 0.13) did not differ significantly from the WT control. Nevertheless, we observed that RelA phosphorylation was reduced after 6 days in culture, compared to the WT control (0.59 ± 0.07, $p < 0.01$) (Fig. 6b). Together, these results show that the protein level of some members of the NF-κB pathway is reduced and RelA phosphorylation is compromised in cultured SMA MNs.

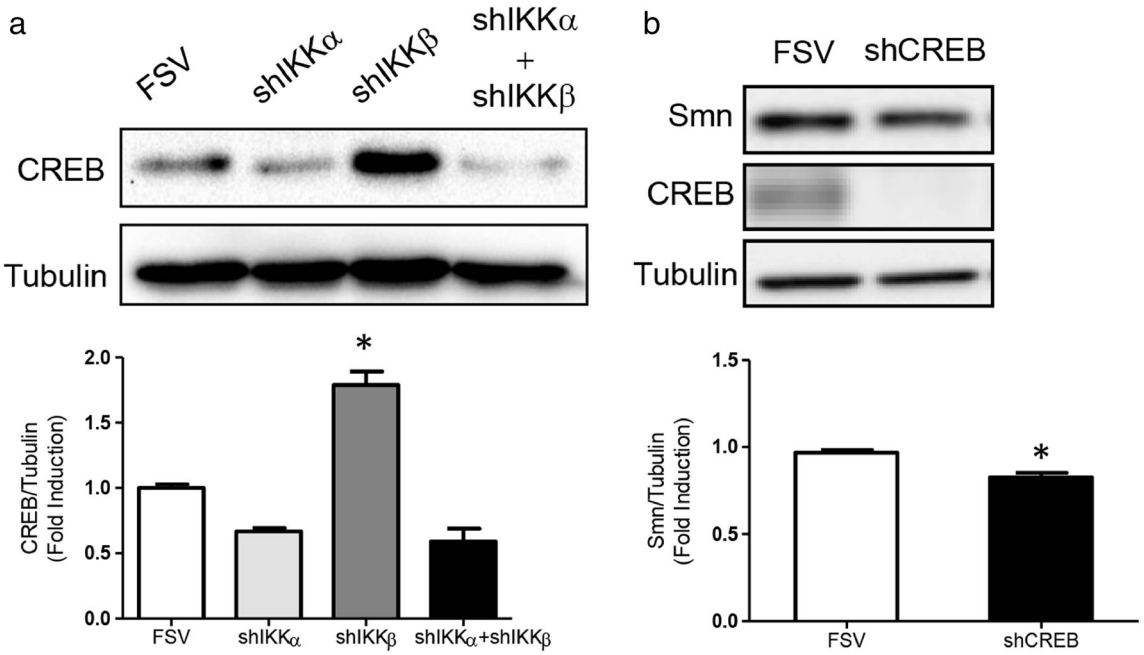

Fig. 5 CREB protein regulation by IKK knockdown. **a** MNs were transduced with lentivirus containing the shIKKα, shIKKβ, shIKKα + shIKKβ or empty vector (FSV) constructs. Five days after transduction, protein lysates were submitted to western blot using an anti-CREB antibody. **b** MNs were transduced with shCREB or FSV and 5 days later, protein extracts were submitted to western blot analysis using an anti-SMN antibody. In **a** and **b**, membranes were reprobed with an anti-α-tubulin antibody. Graph values represent the expression of CREB or Smn vs α-tubulin and correspond to the quantification of three independent experiments ± SEM. Asterisks in **a** indicate significant differences using one-way ANOVA test and Bonferroni's post hoc multiple comparisons ($p < 0.005$). Asterisk in **b** indicates significant differences using Student *t* test ($p < 0.05$)

Discussion

In the present work, we show that the canonical NF-κB pathway regulates Smn protein in a primary culture of isolated mouse embryonic MNs. Reducing protein levels of several members of the NF-κB signalling pathway revealed that these proteins modulate Smn. RelA knockdown was associated with a significant decrease in Smn, but RelB reduction was not. The NF-κB pathway can be activated by two main routes: canonical (or classical) and non-canonical. The canonical pathway is characterized by the activation and translocation of p50/RelA heterodimers to the nucleus, whereas the non-canonical pathway relies on p52/RelB heterodimers [7]. It is known that RelA and RelB heterodimers regulate separate subsets of NF-κB target genes and might therefore regulate diverse cellular functions [29, 30]. Previous studies on MN survival describe this differential effect: RelA knockdown caused MN cell death; RelB knockdown did not [13]. Our present results clearly indicate that canonical NFκ-B pathway inhibition reduces Smn levels in cultured MNs, supporting the involvement of this pathway in the regulation of Smn in these cells. This hypothesis is also supported by the results observed after pharmacological inhibition of NF-κB using SN50, a cell-permeable peptide that blocks the nuclear localization sequence of p50 and prevents its nuclear translocation [25]: SN50 treatment clearly reduced Smn level in cultured MNs; therefore, pharmacological and knockdown experiments have provided evidence that NF-κB activation is involved in Smn stability in MNs.

Inhibition of IκB kinases regulates Smn differentially. Smn protein decreased with IKKα knockdown and increased with IKKβ knockdown. IKKβ is the predominant kinase responsible for IκBα and p105 phosphorylation [31]. In cultured MNs, we described a significant reduction in RelA nuclear translocation and phosphorylation in IKKβ knockdown compared with IKKα knockdown cells, supporting the predominant role of IKKβ in activating the canonical NF-κB pathway in these cells. Previous studies using IKKβ knockout mice demonstrate a clear phenotype similar to RelA knockout, suggesting that IKKβ causes canonical NF-κB pathway activation [31, 32]. Although IKKα and IKKβ share structural and biochemical similarities, different phenotypes in IKKα and IKKβ knockout mice imply distinct physiological roles of the IKK isoforms [33]. They phosphorylate distinct substrates in the cytoplasm and their sub-cellular distribution differs: IKKα can be detected in both the cytoplasm and the nucleus whereas IKKβ is detected predominantly in the cytoplasm [34]. Studies of the nuclear role of IKKα provide evidences for the essential role of this kinase in NF-κB-dependent transcription. Our results are also in accordance with earlier studies of the roles of IKKα and IKKβ in other cellular models, such as chondrocytes and fibroblasts [35, 36], indicating that

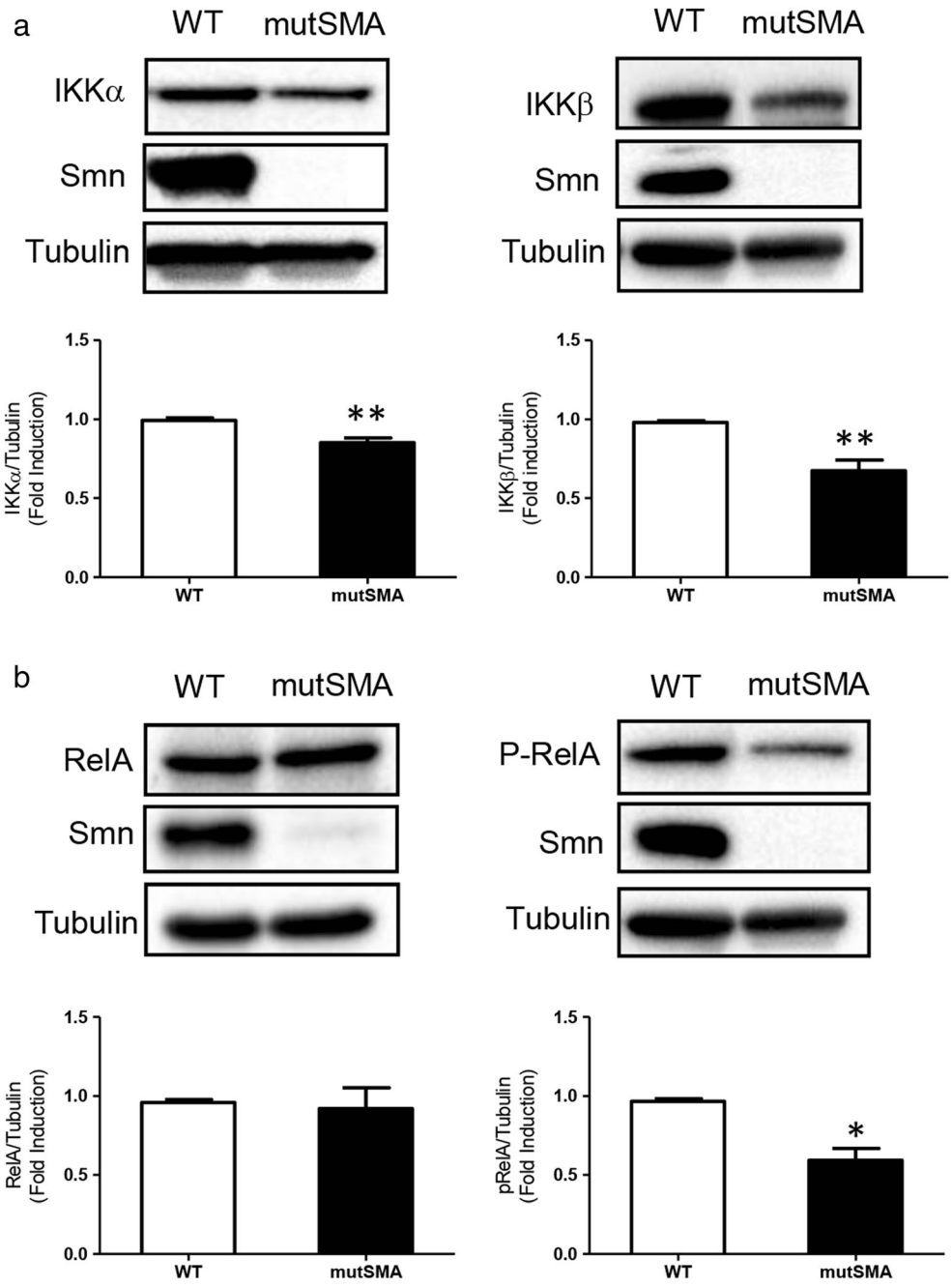

Fig. 6 NF-κB members are modified in SMA-cultured mice spinal-cord MNs. WT and mutSMA isolated MNs were cultured in the presence of NTFs. Six days later, protein extracts were submitted to western blot analysis using the following antibodies: anti-IKKα, anti-IKKβ (**a**), anti-RelA, anti-phosphoRelA (P-RelA) (**b**) and anti-SMN. Membranes were reprobed with an anti-α-tubulin antibody as a loading control. Graph values represent the expression of IKKα, IKKβ, RelA or P-RelA vs α-tubulin and correspond to the quantification of at least three independent experiments ± SEM. Asterisks indicate significant differences using Student *t* test (*$p < 0.05$, **$p < 0.005$)

the differential requirements for IKK in regulating proteins can be an extended process.

The addition of the proteasome inhibitor MG132 to the shIKKα condition prevented Smn reduction. This finding suggests that proteasome activity may be increased in IKKα knockdown cells, which could be the main reason for Smn decrease in this culture condition. It has been previously reported that SMN protein degradation is mediated via the ubiquitin/proteasome pathway, which regulates its cytoplasmic level [27]. In MNs, treatment with proteasome inhibitors increases SMN protein level [37], suggesting that this degradation pathway is involved in SMN stability in these neurons. Therefore, proteasome activity and SMN level may constitute a key point in MN degeneration in SMA. Increased proteasome activity can exacerbate SMN reduction, leading to deterioration in MN function.

On the other hand, the effect of IKKβ knockdown on Smn level occurs through increased *Smn mRNA*. Thus, the absence of IKKβ stimulates Smn transcription. One candidate to produce this effect is CREB. Previous experiments described CREB protein binding to the CRE-II site in the *SMN* promoter as positively regulating *SMN* expression, and treatment with cAMP-elevating agents increased expression of both the full-length and exonΔ7SMN transcript in HeLa cells [28].

Moreover, when activated in SMA spinal cords, CREB binding to its response elements is significantly increased at the level of the SMN2 gene promoter and is correlated with an increase in SMN expression [38]. We measured CREB protein level in IKKα- and IKKβ-reduced cells, and our results showed the increase of CREB in shIKKβ condition and the reduction of CREB in shIKKα. Furthermore, CREB-reduced cells showed a slight, but significant, Smn decrease, indicating that CREB may be regulating SMN protein level in MNs. In conclusion, our results are in accordance with previous reports describing CREB involvement in SMN regulation.

The contribution of IKKs in neurodegenerative disorders has been proposed as a direct interaction of the IKK complex with huntingtin [39] and as regulating the pathogenesis of Huntington disease by inducing cleavage of mutated huntingtin [40]. IKKs phosphorylate huntingtin residues, activating their clearance by proteasomes and lysosomes. SMN complex and SMN itself can be phosphorylated in serine/threonine and lysine residues that regulate the subcellular localization of the SMN complex and its accumulation into the nucleus [41]. PKA can phosphorylate SMN, reducing its degradation in cultured cells [26]. These evidences may lead to a further analysis of the role of SMN phosphorylation, proteasome degradation, and NF-κB pathway. A comprehensive analysis of post-translational modifications such as phosphorylation and increase of proteasome degradation can explain some of the events involved in SMA pathogenesis and the specific vulnerability of spinal cord MNs to SMN protein level. However, studies using SMA patient-derived fibroblasts have not detected alterations in proteasome activity [26], suggesting that proteasome function in SMA patient cells is preserved; nevertheless, pharmacological inhibition of proteasome activity, together with an upregulation of *SMN* gene transcription, increases SMN levels and improves lifespan of SMA mice [42, 43]. Additionally, treatment with sodium butyrate-based compounds ameliorates SMA pathology, possibly by regulating intracellular pathways altered in SMN deficiency and not by regulating SMN protein expression [44]. These observations should help to further define the pathways and allow the identification of more specific targets for therapeutic approaches.

Our results provide new evidences that the NF-κB pathway is modified in MNs of a mouse SMA model. Changes in RelA protein levels were not statistically significant, but the phosphorylation of RelA was reduced in SMA samples. Given recent results supporting the hypothesis that the regulation of altered intracellular pathways may be the future pharmacological treatment of SMA [44, 45], we propose the NF-κB pathway as a candidate to this new therapeutic model.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing financial interests.

References

1. Gallagher D, Gutierrez H, Gavalda N, O'Keeffe G, Hay R, Davies AM (2007) Nuclear factor-kappaB activation via tyrosine phosphorylation of inhibitor kappaB-alpha is crucial for ciliary neurotrophic factor-promoted neurite growth from developing neurons. J Neurosci 27:9664–9669
2. Gutierrez H, O'Keeffe GW, Gavalda N, Gallagher D, Davies AM (2008) Nuclear factor kappa B signaling either stimulates or inhibits neurite growth depending on the phosphorylation status of p65/RelA. J Neurosci 28:8246–8256
3. Albensi BC, Mattson MP (2000) Evidence for the involvement of TNF and NF-kappaB in hippocampal synaptic plasticity. Synapse 35:151–159
4. Nakai M, Qin ZH, Chen JF, Wang Y, Chase TN (2000) Kainic acid-induced apoptosis in rat striatum is associated with nuclear factor-kappaB activation. J Neurochem 74:647–658
5. Kaltschmidt B, Widera D, Kaltschmidt C (2005) Signaling via NF-kappaB in the nervous system. Biochim Biophys Acta 1745:287–299
6. Mincheva-Tasheva S, Soler RM (2013) NF-κB signaling pathways: role in nervous system physiology and pathology. Neuroscientist 19:175–194
7. Perkins ND (2007) Integrating cell-signalling pathways with NF-kappaB and IKK function. Nat Rev Mol Cell Biol 8:49–62
8. Christian F, Smith EL, Carmody RJ (2016) The regulation of NF-κB subunits by phosphorylation. Cell 5
9. Bui NT, Livolsi A, Peyron JF, Prehn JH (2001) Activation of nuclear factor kappaB and Bcl-x survival gene expression by nerve growth factor requires tyrosine phosphorylation of IkappaBalpha. J Cell Biol 152:753–764
10. Saha RN, Liu X, Pahan K (2006) Up-regulation of BDNF in astrocytes by TNF-alpha: a case for the neuroprotective role of cytokine. J NeuroImmune Pharmacol 1:212–222
11. Simpson CS, Morris BJ (2000) Regulation of neuronal cell adhesion molecule expression by NF-kappa B. J Biol Chem 275:16879–16884
12. Rojo AI, Salinas M, Martín D, Perona R, Cuadrado A (2004) Regulation of Cu/Zn-superoxide dismutase expression via the phosphatidylinositol 3 kinase/Akt pathway and nuclear factor-kappaB. J Neurosci 24:7324–7334
13. Mincheva S, Garcera A, Gou-Fabregas M, Encinas M, Dolcet X, Soler RM (2011) The canonical nuclear factor-kappa B pathway regulates cell survival in a developmental model of spinal cord Motoneurons. J Neurosci 31:6493–6503
14. Bhakar AL, Tannis LL, Zeindler C, Russo MP, Jobin C, Park DS, MacPherson S, Barker PA (2002) Constitutive nuclear factor-kappa B activity is required for central neuron survival. J Neurosci 22:8466–8475
15. Kaltschmidt B, Uherek M, Volk B, Baeuerle PA, Kaltschmidt C (1997) Transcription factor NF-kappaB is activated in primary neurons by amyloid beta peptides and in neurons surrounding early

plaques from patients with Alzheimer disease. Proc Natl Acad Sci U S A 94:2642–2647
16. Cardoso SM, Oliveira CR (2003) Inhibition of NF-kB renders cells more vulnerable to apoptosis induced by amyloid beta peptides. Free Radic Res 37:967–973
17. Lefebvre S, Burglen L, Reboullet S, Clermont O, Burlet P, Viollet L, Benichou B, Cruaud C et al (1995) Identification and characterization of a spinal muscular atrophy-determining gene. Cell 80: 155–165
18. Lefebvre S, Burlet P, Liu Q, Bertrandy S, Clermont O, Munnich A, Dreyfuss G, Melki J (1997) Correlation between severity and SMN protein level in spinal muscular atrophy. Nat Genet 16:265–269
19. Sumner CJ (2006) Therapeutics development for spinal muscular atrophy. NeuroRx 3:235–245
20. Arce V, Garces A, de Bovis B, Filippi P, Henderson C, Pettmann B, deLapeyriere O (1999) Cardiotrophin-1 requires LIFRbeta to promote survival of mouse motoneurons purified by a novel technique. J Neurosci Res 55:119–126
21. Gou-Fabregas M, Garcera A, Mincheva S, Perez-Garcia MJ, Comella JX, Soler RM (2009) Specific vulnerability of mouse spinal cord motoneurons to membrane depolarization. J Neurochem 110:1842–1854
22. Perez-Garcia MJ, Cena V, de Pablo Y, Llovera M, Comella JX, Soler RM (2004) Glial cell line-derived neurotrophic factor increases intracellular calcium concentration. Role of calcium/calmodulin in the activation of the phosphatidylinositol 3-kinase pathway. J Biol Chem 279:6132–6142
23. Encinas M, Rozen EJ, Dolcet X, Jain S, Comella JX, Milbrandt J, Johnson EM Jr (2008) Analysis of Ret knockin mice reveals a critical role for IKKs, but not PI 3-K, in neurotrophic factor-induced survival of sympathetic neurons. Cell Death Differ 15: 1510–1521
24. Dolcet X, Llobet D, Encinas M, Pallares J, Cabero A, Schoenenberger JA, Comella JX, Matias-Guiu X (2006) Proteasome inhibitors induce death but activate NF-kappaB on endometrial carcinoma cell lines and primary culture explants. J Biol Chem 281:22118–22130
25. Lin YZ, Yao SY, Veach RA, Torgerson TR, Hawiger J (1995) Inhibition of nuclear translocation of transcription factor NF-kappa B by a synthetic peptide containing a cell membrane-permeable motif and nuclear localization sequence. J Biol Chem 270:14255–14258
26. Burnett BG, Muñoz E, Tandon A, Kwon DY, Sumner CJ, Fischbeck KH (2009) Regulation of SMN protein stability. Mol Cell Biol 29:1107–1115
27. Chang HC, Hung WC, Chuang YJ, Jong YJ (2004) Degradation of survival motor neuron (SMN) protein is mediated via the ubiquitin/proteasome pathway. Neurochem Int 45:1107–1112
28. Majumder S, Varadharaj S, Ghoshal K, Monani U, Burghes AH, Jacob ST (2004) Identification of a novel cyclic AMP-response element (CRE-II) and the role of CREB-1 in the cAMP-induced expression of the survival motor neuron (SMN) gene. J Biol Chem 279:14803–14811
29. Kaltschmidt B, Heinrich M, Kaltschmidt C (2002) Stimulus-dependent activation of NF-kappaB specifies apoptosis or neuroprotection in cerebellar granule cells. NeuroMolecular Med 2:299–309
30. Chu ZL, McKinsey TA, Liu L, Gentry JJ, Malim MH, Ballard DW (1997) Suppression of tumor necrosis factor-induced cell death by inhibitor of apoptosis c-IAP2 is under NF-kappaB control. Proc Natl Acad Sci U S A 94:10057–10062
31. Li ZW, Chu W, Hu Y, Delhase M, Deerinck T, Ellisman M, Johnson R, Karin M (1999) The IKKbeta subunit of IkappaB kinase (IKK) is essential for nuclear factor kappaB activation and prevention of apoptosis. J Exp Med 189:1839–1845
32. Li Q, Estepa G, Memet S, Israel A, Verma IM (2000) Complete lack of NF-kappaB activity in IKK1 and IKK2 double-deficient mice: additional defect in neurulation. Genes Dev 14:1729–1733
33. Liu F, Xia Y, Parker AS, Verma IM (2012) IKK biology. Immunol Rev 246:239–253
34. Huang WC, Hung MC (2013) Beyond NF-κB activation: nuclear functions of IκB kinase α. J Biomed Sci 20:3
35. Olivotto E, Borzi RM, Vitellozzi R, Pagani S, Facchini A, Battistelli M, Penzo M, Li X et al (2008) Differential requirements for IKKalpha and IKKbeta in the differentiation of primary human osteoarthritic chondrocytes. Arthritis Rheum 58:227–239
36. Lamberti C, Lin KM, Yamamoto Y, Verma U, Verma IM, Byers S, Gaynor RB (2001) Regulation of beta-catenin function by the IkappaB kinases. J Biol Chem 276:42276–42286
37. Locatelli D, Terao M, Kurosaki M, Zanellati MC, Pletto DR, Finardi A, Colciaghi F, Garattini E et al (2015) Different stability and proteasome-mediated degradation rate of SMN protein isoforms. PLoS One 10:e0134163
38. Branchu J, Biondi O, Chali F, Collin T, Leroy F, Mamchaoui K, Makoukji J, Pariset C et al (2013) Shift from extracellular signal-regulated kinase to AKT/cAMP response element-binding protein pathway increases survival-motor-neuron expression in spinal-muscular-atrophy-like mice and patient cells. J Neurosci 33:4280–4294
39. Khoshnan A, Ko J, Tescu S, Brundin P, Patterson PH (2009) IKKalpha and IKKbeta regulation of DNA damage-induced cleavage of huntingtin. PLoS One 4:e5768
40. Thompson LM, Aiken CT, Kaltenbach LS, Agrawal N, Illes K, Khoshnan A, Martinez-Vincente M, Arrasate M et al (2009) IKK phosphorylates Huntingtin and targets it for degradation by the proteasome and lysosome. J Cell Biol 187:1083–1099
41. Husedzinovic A, Oppermann F, Draeger-Meurer S, Chari A, Fischer U, Daub H, Gruss OJ (2014) Phosphoregulation of the human SMN complex. Eur J Cell Biol 93:106–117
42. Kwon DY, Motley WW, Fischbeck KH, Burnett BG (2011) Increasing expression and decreasing degradation of SMN ameliorate the spinal muscular atrophy phenotype in mice. Hum Mol Genet 20:3667–3677
43. Foran E, Kwon DY, Nofziger JH, Arnold ES, Hall MD, Fischbeck KH, Burnett BG (2016) CNS uptake of bortezomib is enhanced by P-glycoprotein inhibition: implications for spinal muscular atrophy. Neurobiol Dis 88:118–124
44. Butchbach ME, Lumpkin CJ, Harris AW, Saieva L, Edwards JD, Workman E, Simard LR, Pellizzoni L et al (2016) Protective effects of butyrate-based compounds on a mouse model for spinal muscular atrophy. Exp Neurol 279:13–26
45. Tisdale S, Pellizzoni L (2015) Disease mechanisms and therapeutic approaches in spinal muscular atrophy. J Neurosci 35:8691–8700