Bilateral crosstalk of rho- and extracellular-signal-regulated-kinase (ERK) pathways is confined to an unidirectional mode in spinal muscular atrophy (SMA)

Niko Hensel a,b, Inga Stockbrügger a, Sebastian Rademacher a,b, Natasha Broughton a, Hella Brinkmann a, Claudia Grothe a,b, Peter Claus a,b,c,*

* Institute of Neuroanatomy, Hannover Medical School, 30625 Hannover, Germany
† Center for Systems Neuroscience (ZSN), 30559 Hannover, Germany
‡ Niedersachsen Research Network on Neuroinfection (N-RENNT), Germany

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Rho-kinase (ROCK) as well as extracellular signal regulated kinase (ERK) control actin cytoskeletal organization thereby regulating dynamic changes of cellular morphology. In neurons, motility processes such as axonal guidance and neurite outgrowth demand a fine regulation of upstream pathways. Here we demonstrate a bilateral ROCK–ERK information flow in neurons. This process is shifted towards an unidirectional crosstalk in a model of the neurodegenerative disease Spinal Muscular Atrophy (SMA), ultimately leading to neurite outgrowth dysregulations. As both pathways are of therapeutic relevance for SMA, our results argue for a combinatorial ROCK/ERK-targeting as a future treatment strategy.

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1. Introduction

The neurodegenerative disease Spinal Muscular Atrophy (SMA) is characterized by proximal muscle weakness and atrophy induced by degeneration of the α-motoneurons in the spinal cord. SMA is of mono-genetic origin; all patients suffer from deletions or mutations of the Survivin (Sur) gene [1,2]. Humans, however, exhibit the very similar Smn gene, which only differs in one base-pair exchange within exon 7. Although translational silent, this mutation locates to an exonic splice enhancer region leading to misspliced mRNA and only 10% functional full-length protein, derived from Smn2 when compared to Smn1 [3]. Thus, Smn2 only partially rescues SMA-phenotype and as a consequence the severity of disease correlates with Smn2 copy number. Three main subpopulations of patients can be discriminated depending on their motor abilities [4]. Type 1 or severely affected patients are never able to walk, type 2-patients never able to sit while type 3-patients are able to walk but display progressive muscular weakness [5].

The examination of interaction partners and functions of the SMN-protein is a strategy to elucidate the molecular pathomechanism of SMA. Despite the well characterized function of SMN in spliceosomal snRNP assembly [6], there are only few models concerning less basal and more neuronal-specific functions. Interestingly, SMN is involved in axonal transport of mRNAs important for cytoskeletal metabolism such as β-actin [7,8]. Additionally, we and others have shown a neuron-specific role of SMN in regulation of actin dynamics and neurite outgrowth [9–13]. SMN directly binds to profilin–2a, an actin binding protein expressed in neuronal tissues, and thereby regulates the Rho-kinase (ROCK) pathway. Functionally, SMN reduction leads to dysregulations in ROCK-activity resulting in reduced neurite outgrowth in sympathetic neuron-like PC12 cells [9]. Despite the ROCK-pathway controlling neurite outgrowth, we could show an upregulation of the ERK-pathway in the motoneuron-like NSC34 cell line [14]. ERK, however, is a well known agonist of neurite outgrowth, indicating an antagonistic role in SMA compared to ROCK-pathway dysregulations [15,16]. However, both pathways are of therapeutic relevance; ROCK- as well as ERK-inhibition lead to enhanced survival of SMA-model mice [17–19]. Thus, we further analyzed ROCK- and ERK-pathway dysregulations in NSC34 cells. In this study, we demonstrate that both pathways are bi-laterally interconnected in neurons. The architecture of the ROCK–ERK network allows bi-directional flow of information in its default state to regulate neuronal differentiation. In SMA, however, the activity of the pathways becomes dysregulated and the crosstalk restricted towards an uni-directional mode. On a functional level, this
results in changes of neurite outgrowth. Furthermore, this crosstalk mechanism points towards new strategies for future SMA-treatments.

2. Material and methods

2.1. Cell culture and transfection

NSC34 and PC12 cells were incubated at 37 °C in humidified atmosphere with 5% CO2. NSC34 cells were grown in Dulbecco’s modified Eagle medium (DMEM) containing low amounts of glucose, 5% (v/v) fetal calf serum (FCS), 200 mM l-glutamine and 100 U/ml penicillin and 0.1 mg streptomycin. For differentiation, medium was changed to low serum condition (1% (v/v) FCS) 24 h after seeding and incubated for another 72 h before analysis. To assure compatibility between biochemical and neurite outgrowth assays, cells were seeded at same densities for outgrowth assays, transfections with siRNAs and plasmids, application of inhibitors and changing to differentiation medium were performed simultaneously. For crosstalk analysis, inhibitors were applied 2 h prior to lysis, while induction with agonists, fibroblast growth factor-2 (FGF-2, 50 ng/µl), was performed 10 min prior to lysis. For transfection, Metafectene Pro (Biontex Laboratories GmbH) was applied according to the manufacturer’s protocol. NSC34 cells were transfected with three different siRNAs against murine SMN and one scrambled control siRNA (scr1), respectively [14]. To rule out any off-target effects, two more scrambled control siRNAs were included: scr2 (AGAUAUAUAUACG UAUAUA), and scr4 (AUAGCAACGAAAGCAACAAG), PC12 cells were maintained in high glucose DMEM containing 10% (v/v) horse serum and 5% (v/v) fetal calf serum (FCS), 200 mM l-glutamine and 100 U/µl penicillin and 0.1 mg streptomycin (proliferation medium). For transfections with siRNAs, Lipofectamine 2000 (Invitrogen) was used according to manufacturer’s suggestions: Cells were detached, suspended in proliferation medium, mixed with transfection solution and seeded onto poly-L-lysine (PLL) coated wells. After 6 h, medium was changed to differentiation medium containing 1% (v/v) horse serum and maintained in this medium for 72 h prior to analysis. Three different siRNAs against rat SMN were applied, si4 (CAGAGUAUAAG ACA-CACGCA), si5 (AGAAGCGUGACAUUGUG-GA) and si6 (AACCUG CUAAAGAAUA), and compared with scrambled siRNAs. Recombinant human FGF-2 (PeproTech) and murine nerve growth factor (NGF, Promega) were employed at final concentrations of 50 ng/ml. Lysophosphatic acid (LPA) was used at a concentration of 10 µM. Inhibitors (Calbiochem) were applied at following concentrations: Y27632 for Rho-kinase (50 µM), FR180204 for ERK (100 µM) and PD173074 for fibroblast growth factor receptor 1 (FGFR1) (200 nM).

2.2. Western blot

Western blot analysis was conducted as described previously [14]. The following antibodies were used: α-SMN (BD Biosciences, 1:4000), α-c-tubulin (Santa Cruz, 1:2000), α-P-cofilin-Ser3 (Santa Cruz, 1:1000), α-cofilin (Cell Signaling, 1:1000), α-P-ERK (T202,Y204) (Cell Signaling, 1:1000), α-ERK (Cell Signaling, 1:1000), α-P-p90RSK (Cell Signaling, 1:1000), α-P-c-Raf(S259) (Cell Signaling, 1:500), α-c-Raf(S338) (Cell Signaling, 1:500), α-c-Raf (Cell Signaling, 1:500), α-P-Mek/(Ser217/221) (Cell Signaling, 1:1000), and α-Mek (Cell Signaling, 1:2000). Densitometric quantification was performed using Labimage 1D-software (Intas) and statistically analyzed by a two-way ANOVA with Bonferroni post tests.

2.3. Neurite outgrowth assays

To ensure comparability to western blot analyses, cells were seeded at same densities on poly-L-lysine (PLL)-coated coverslips. For tracing of single neurites in this relatively dense monolayer, cells were transfected with plasmids coding for either Discosoma Red (DsRed2) or enhanced green fluorescent protein (EGFP). Transfection in differentiation medium ensured both, a high transfection rate for siRNAs and a low transfection rate for plasmids to label cells for subsequent neurite tracing. NSC34 cells tend to differentiate spontaneously already under proliferative conditions. However, fluorescent cells undergo outgrowth during 72 h of differentiation allowing evaluation of neurite outgrowth rather than neurite maintenance. In addition to reporter plasmids coding for fluorescent proteins, full length human pSMN1-294-EGFP was transfected [9,20]. After 72 h of differentiation, cells were fixed with 4% (w/v) paraformaldehyde in PBS, washed and stained against SMN (BD Biosciences, 1:500). Blocking as well as staining was conducted in 0.3% (v/v) Triton-X100 and 3% (v/v) Normal Goat Serum (NGS) in phosphate buffered saline (PBS). Images were obtained on an Olympus BX60-microscope with fixed settings ensuring compatibility of fluorescence intensities between experiments. Measurement of neurite lengths was performed by using the ImageJ-based neurite tracer NeuronJ [21]. Ten randomly selected neurites distributed over the whole coverslip were measured and averaged for each biologically independent repetition. To avoid pseudo-replication, statistical analysis was performed on averaged values. Microscopy and measurements of neurite lengths were carried out blinded.

3. Results

3.1. Crosstalk of ROCK- and ERK-pathways

ROCK is an antagonist of neurite outgrowth while ERK typically induces neurite elongation [15,16]. We have previously demonstrated dysregulations of both pathways in SMA models [9,14]. Therefore, we analyzed the possibility of a mutual interaction of ROCK- and ERK-signaling. Bilateral crosstalk of these pathways has not been analyzed before and was elucidated here by a systematic approach in a cellular model of SMA in motoneuron-like NSC34 cells [22]. We triggered ERK-signaling by fibroblast growth factor-2 (FGF-2) [23] and ROCK-signaling by lysophosphatic acid (LPA). LPA is a common upstream activator of RhoA which further activates ROCK signaling [24]. Treatment of cells with the ROCK-inhibitor Y27632 (Y) and evaluation of phospho-ERK-levels provided information about a ROCK- to ERK-crosstalk. Vice versa, application of ERK-inhibitor FR180204 (FR) and evaluation of ROCK-activity by measuring phosphorylation of its indirect downstream target cofilin allowed for ERK to ROCK crosstalk analysis. Additionally, we included SMN-siRNA transfections to investigate the SMN-dependancy of the crosstalk. After SMN knock-down, we observed significantly enhanced ERK-activity compared to control transfections with scrambled siRNA in all conditions (Fig. 1A, B, lanes 1–9) consistent with our previous results [14] except for control cells treated with ERK-inhibitor. As expected, treatment of cells with fibroblast growth factor-2 (FGF-2) generally increased phosphorylation levels of ERK and maintained the significant differences between SMN knock-down and control conditions (Fig. 1A, B, lanes 7–9). Importantly, concomitant FGF-2 incubation and ROCK-inhibition caused enhanced ERK-activation (Fig. 1B, lanes 7 and 8), indicating the presence of ROCK to ERK crosstalk. This communication of the pathways was not dependent on SMN since we could not observe any change of ratios in scrambled control or siRNA-treated cells (Fig. 1B, FGF-2, lanes 7 and 8).

Induction of the ROCK pathway by LPA did not inhibit ERK-activity (Fig. 1B, lanes 4 compared to 1). A simultaneous FGF-2 induction and ERK-inhibition by FR180204 caused strong phospho-ERK signals (Fig. 1B, lanes 7 and 9). Since the ERK-inhibitor acts downstream of ERK by restricting ERK-kinase activity rather than impeding ERK-phosphorylation itself, this observation hints for an inhibition of a negative feedback-loop. This mechanism, however, only forms when triggered with FGF-2 as compared to the control indicating the need of enhanced ERK activation (Fig. 1B, lanes 9 versus 3).
Downstream of ROCK, cofilin displayed enhanced phosphorylation under SMN knock-down which could be suppressed by ROCK-inhibition, revealing an increased ROCK activity in NSC34 cells (Fig. 1C, lanes 1–2). As expected, ROCK pathway activation by LPA increased phospho-cofilin levels (Fig. 1C, lanes 4) and ROCK inhibitor Y27632 treatment reduced the signal to control values (Fig. 1C, lanes 5 and 2). FGF-2 did not show any influence on ROCK activity (Fig. 1C, lanes 7–9). Treatment with ERK-inhibitor FR180204 induced phosphorylation of cofilin (Fig. 1C, lanes 3 versus 1) indicating a crosstalk from ERK to ROCK pathway. The crosstalk is dependent on SMN since phospho-cofilin levels did not change in SMN knock-down cells with or without ERK-inhibitor (Fig. 1C, siRNA-treated cells in lanes 3 and 1). Independent of specific pathway activation by LPA or FGF-2, ERK exerted an inhibitory function on ROCK which became impeded under SMN knock-down (Fig. 1C, lanes 1–9).

In conclusion, we demonstrated ROCK to ERK and vice versa bilateral crosstalk in this neuronal model. SMN reduction impedes ERK to ROCK crosstalk resulting in uni-directional communication from ROCK to ERK and simultaneous hyper-activity of ERK and ROCK.

3.2. ROCK input node within ERK-pathway

To mechanistically elucidate the crosstalk, we decided to blot for signaling intermediates upstream of ERK. MAPK/ERK kinases (Mek) directly signal to ERK upon activation by its upstream effector proto-oncogene c-RAF (cRaf) [25]. Interestingly, a direct interaction of ROCK with cRaf has been reported before [26]. Moreover, a proteomic screening for ROCK substrates identified cRaf serine residue 259 as a potential ROCK target [27]. As serine 259 is an inhibitory phospho-site [28] it is thus possible, that ROCK indirectly inhibits ERK by phosphorylation of cRaf at serine 259 leading to its subsequent inactivation. To test this hypothesis, NSC34 cells were treated with ROCK-inhibitor Y27632 and ERK inhibitor FR180204, respectively, and compared with DMSO-treated control cells (Fig. 2). Additionally, cells were induced with...
FGF-2 as this provided the strongest effects with regard to crosstalk (Fig. 1B, lanes 7–9). The bilateral crosstalk was confirmed by enhanced phospho-ERK signal intensities under Y27632 treatment as well as an increase of phospho-cofilin levels under FR180204 treatment (Fig. 2A). Phospho-Mek levels were elevated by ROCK- as well as ERK inhibition (Fig. 2A, B) recapitulating the behavior of its downstream target ERK (Fig. 1B, lanes 7–9). Thus, the negative feedback-loop regulating ERK activity and more importantly, the ROCK to ERK crosstalk, were dependent upon the ERK upstream activator Mek. However, evaluation of phospho-cRaf levels for serine 338, reflecting its activation status [29], as well as negative regulatory phospho-site serine 259, did not reveal any regulations upon inhibitor treatment. Thus, the ROCK input node within the cRaf/Mek/ERK cascade can be confined to a position downstream of cRaf either by altering the cRaf-Mek binding or by directly regulating the phospho-status of Mek.

3.3. Neurite outgrowth and dysregulations of pathways

Our crosstalk analysis revealed an enhanced ROCK-activity in SMN knock-down NSC34 cells. We could previously show enhanced and sustained ERK-phosphorylation in SMN-reduced NSC34 cells [14]. However, to examine signaling cascades independent of expression changes or medium effects, our crosstalk analysis was performed by two-hour incubation in starvation medium with inhibitors followed by short induction with LPA or FGF-2, respectively. These conditions were employed after successful knock-down of SMN for a period of three days in NSC34 cells (Fig. S1). In contrast, neurite outgrowth assays require conditioned medium with serum for neuronal differentiation. As shown previously, ERK becomes hyper-activated under this condition with SMN knock-down [14]. Activity of the ROCK-pathway, however, was still unknown. Therefore, we next analyzed the ROCK-activity in NSC34 cells in differentiating conditions. Phospho-cofilin levels were upregulated indicating ROCK hyper-activation (Fig. 3A, B) in knock-down conditions.

In PC12 cells, we have previously shown that after a three-day knock-down of SMN, ROCK activity was enhanced towards its downstream target profilin-2a, while its activity was reduced with regard to cofilin phosphorylation [9]. This pattern of dysregulation could be linked to morphological changes as ROCK inhibition rescued SMN knock-down induced neurite outgrowth defects [9]. Although NSC34 and PC12 cells displayed differential regulation of phospho-cofilin

Fig. 2. Crosstalk upstream of ERK. NSC34 cells were treated with DMSO, ROCK inhibitor Y27632 and ERK inhibitor FR180204 for 2 h and induced with FGF-2 for 10 min prior to lysis. (A) Western blots with antibodies against phospho-ERK (P-ERK), ERK, phospho-cofilin (P-cof), α-tubulin (α-tub), phospho-Mek (P-Mek) as well as phospho-cRaf sites serine 338 (P-cRaf(S338)) and serine 259 (P-cRaf(S259)) in comparison with total cRaf. (B–D) Densitometrical analyses of phospho-protein signal intensities for (B) phospho-Mek, (C) phospho-cRaf(S338) and (D) phospho-cRaf(S259); n = 3, student’s t-test (** p < 0.01, ns (non significant)).

Fig. 3. Cell specificity and ROCK- and ERK-pathway dysregulations in conditioned media. NSC34 cells (A, B) as well as PC12 cells (C, D) were transfected with siRNAs against SMN (si) and scrambled control siRNAs (scr) and kept in differentiation medium for three days. (A) Western blot of NSC34 cell lysates with phospho-cofilin (P-cof), cofilin (cof), SMN, and α-tubulin (α-tub); (B) Western blot of PC12 cell lysate with phospho-ERK (P-ERK) in comparison to ERK; (C) Western blot of PC12 cell lysate with phospho-ERK (P-ERK) in comparison to ERK; (D) Densitometrical analysis of P-ERK (D) on western-blots. n = 5, student’s t-test (* p < 0.05, ns (non significant)).
levels, both showed dysregulations of the ROCK pathway. To evaluate whether ERK-dysregulation is also conserved in both cell types, we next analyzed ERK activity in PC12 cells. PC12 cells displayed no regulation of the ERK-pathway (Fig. 3C, D). In contrast to PC12 cells in which ROCK is the only affected pathway leading to neurite outgrowth defects, NSC34 cells display a unique dysregulation pattern of steady ROCK- and ERK-hyper-activation.

3.4. Enhanced neurite outgrowth in SMN-deprived NSC34 cells

As cell density could have a significant effect on pathway activation controlling neurite outgrowth, we decided to seed the same number of cells as used in western blot analyses. For tracing of single neurites in the dense monolayer, we cotransfected a plasmid coding for Discosoma red protein (DsRed) highlighting neuronal processes (Fig. 4A–D). Additionally, we monitored knock-down in individual cells by immunocytochemical staining against SMN (Fig. 4A′, B′ and insets). Interestingly, the knock-down of SMN induces a strong outgrowth, while SMN-overexpression had no effect (Fig. 4E, F). We validated our scrambled control siRNA for off-target effects by including additional scrambled control siRNAs. However, we could not find any negative effect of control siRNA transfections on outgrowth (Fig. S2A). As coating might have a substantial influence on adhesion and neurite outgrowth, we therefore checked whether there is any differential effect between SMN knock-down and control conditions on enhanced neurite outgrowth on different growth-permissive substrates (Fig. S2B). No effect on outgrowth could be observed between poly-l-lysine (PLL, also used in previous experiments), rat-tail collagen, polyornithine/fibronectin (Po/Fi) as well as laminin (Po/La).

![Fig. 4](image).

**Fig. 4.** Neurite outgrowth after SMN knock-down and over-expression. (A–D″) NSC34 cells were transfected with siRNA against SMN (si) or scrambled control RNA (scr) (A, B) as well as plasmids coding for SMN-EGFP or EGFP alone (C, D). For neurite tracing, pDsRed2 was co-transfected in all conditions (A–D). SMN knock-down was monitored by immunocytochemistry with an antibody against SMN and detection using an Alexa 488-conjugated secondary antibody (A′, B′). (E) Quantification and statistical analysis of relative neurite lengths after transfection of NSC34 cells with scrambled (control) or siRNA against SMN. (F) Overexpression of SMN did not show changed neurite outgrowth compared to pEGFP-transfected control cells. For E–F, n = 6, paired t-test (* p < 0.05, ns (non significant)).
3.5. ERK-pathway causes enhanced neurite outgrowth in SMN-deprived NSC34 cells

As both ERK and ROCK pathways typically act antagonistically on neurite outgrowth [15,16], we aimed to elucidate how NSC34 cells functionally integrate these signals with regard to neuronal differentiation as a functional consequence. To investigate the involvement of the different pathways in neurite outgrowth of NSC34 cells, we next applied specific inhibitors to the cultures. By FGF-receptor (FGFR)-inhibition in NSC34 cells, we could previously link the ERK hyper-activation after SMN knock-down to FGFR activation [14]. In contrast to the signaling approach as conducted in the crosstalk analysis, in which inhibitors were kept for two hours on cells, a morphological outcome such as neurite length demands prolonged inhibitor incubation. Thus, we first tested the efficacy of the ROCK-inhibitor Y27632, the ERK-inhibitor FR180204 and the FGFR-inhibitor PD173074 during the whole period of differentiation (Fig. 5A–D). In this kinetic approach, different periods of inhibitor incubation were analyzed, without changing the total differentiation time of the cells. This allowed for differentiation-dependent assessment of inhibitor efficacy as needed for morphological evaluations (Fig. 5A). Induction of ROCK-pathway by LPA and FGFR/ERK-pathway by FGF-2 for ROCK- and FGFR/ERK-inhibitor evaluation, respectively, was performed 10 min prior to lysis. The effect of ERK-inhibitor FR180204 was elucidated by analysis of phosphorylated p90RSK, an ERK downstream target (Fig. 5C). Importantly, all three inhibitors displayed efficient activity for the longest incubation period of 72 h (Fig. 5B–D). Therefore, we have used a single application of inhibitors at day 0 for subsequent analysis of neuronal differentiation in outgrowth assays (Fig. 5E).

Neurite outgrowth in SMN knock-down cells was completely equalized compared to control conditions by ERK-inhibition with FR180204 (Fig. 5F–L). While scrambled control-transfected cells did not show reduction in neurite lengths between vehicle DMSO- and ERK-inhibitor-treatment, SMN knock-down cells were strongly affected (Fig. 5L). To rule out any indirect effects due to changes in cell density by proliferation or apoptosis, we counted cells by DAPI-staining (Fig. S3A). However, we observed no statistically significant changes in cell density. The ERK-pathway is known to affect primary differentiation as well as outgrowth [16]. Therefore, we counted differentiated cells with neurites at least as long as one cell diameter and could observe the same changes in this initiation of differentiation as seen before for the outgrowth of neurites longer than 40 μm (Fig. S3B). Although ERK hyper-

![Fig. 5. Modulation of SMN-dependent neurite outgrowth by different inhibitor treatments.](image-url)
phosphorylation induced by SMN knock-down was impeded by FGFR-inhibition [14], we could not observe any effect on neurite outgrowth (Fig. 5L). This indicates a more general compensatory mechanism which might not necessarily be dependent on FGFR upregulation. ROCK inhibition only resulted in a slight enhancement of neurite lengths in control cells not reaching statistical significance due to low basal ROCK activity in NSC34 cells (Fig. 5L). ROCK-inhibition only revealed minor effects on phospho-collin levels, when comparing control cells with inhibited cells (Fig. 5D). Importantly, ROCK-inhibition did not affect SMN siRNA-treated cells (Fig. 5L). Thus, ROCK hyper-activity, as observed on the biochemical level, was completely overcompensated by ERK-activity on the functional level. Moreover, a compensatory mechanism in which enhanced ROCK-activity directly induced ERK-activation can be excluded as this would result in the same pattern as observed with ERK-inhibitor treatment. The results indicate a preferential effect of ERK-inhibition on neurite outgrowth of NSC34 cells in SMN knock-down conditions.

4. Discussion

In this study, we demonstrate for the first time that ROCK- as well as ERK-pathways are bilaterally interconnected in neurons (Fig. 6). Communication of both pathways turns to an unidirectional mode in a neuronal model of SMA. By default, both pathways inhibit each other according to their antagonistic functions: While ROCK mainly inhibits neurite outgrowth, ERK is a well described agonist of neurite elongation [15,16]. To our knowledge, an ERK to ROCK crosstalk had not been described on the post-translational level so far. However, ERK down-regulates expression of ROCK-upstream activator RhoA via its gene target fra-1 in non-neuronal cells [30]. Furthermore, cells displaying a hyperactive isoform of the ERK-upstream agonist Ras show an ERK-dependent reduction in ROCK-protein expression [31], demonstrating ERK-induced inhibition of ROCK expression. An ERK-independent inhibitory binding of cRaf to ROCK has been described [26,32].

What is the molecular mechanism of ROCK to ERK pathway crosstalk? We could not observe any involvement of cRaf-phospho-sites within the cRaf/Mek/ERK cascade. In contrast, Mek displayed changes consistent with phosphorylation of its downstream target ERK. Thus, the ROCK input node within the ERK pathway can be localized downstream of cRaf. This includes a crosstalk mechanism involving cRaf independent of its phospho-status: It is possible that cRaf is sequestered from Mek due to a ROCK-activity dependent competitive binding of cRaf to ROCK. However, further efforts have to be undertaken to clarify the exact mechanism. An unidirectional crosstalk of the ROCK-pathway with ERK on the post-translational level has been reported in vitro: In agreement with our findings, an inhibition of ROCK led to an enhanced ERK-activation in the presence of FGF-1 in PC12 cells [32]. ROCK-inhibition of retinal ganglion cells treated with ERK upstream activator ciliary neurotrophic factor (CNTF) further increased phospho-ERK levels implicating a ROCK to ERK crosstalk. Accordingly, combinatory treatment of a crushed nerve model with CNTF and ROCK-inhibitor led to additive effects on neurite outgrowth and regeneration in vivo [33].

However, only the ERK to ROCK communication was SMN-dependent indicating a specific role of SMN-in the pattern of pathway dysregulations (Fig. 6). We previously reported an enhanced activation of ERK caused by an upregulation of FGFR1 in a NSC34 cell-based knockdown model for SMA. As demonstrated here, NSC34 cells additionally displayed an increased ROCK-activity which cannot be regulated by ERK due to disturbed crosstalk. This leads to the conclusion that enhanced ROCK-activity is not directly caused by increased ERK-activity pinpointing towards a still uncharacterized upstream mechanism. As we could show that ERK is in principle capable of regulating ROCK but fails for that in SMN-deprived cells, it is possible that ERK activity is compensationally upregulated as a reaction to ROCK-hyperactivity. The putative mechanism of this increased ERK-activity in SMA could be a regulation of its upstream factors. Concomitant with that, we could previously link FGFR1 up-regulation with ERK hyper-activity in mouse and cellular SMA models [14].

On the functional level, SMN knock-down in NSC34 cells resulted in an enhanced neurite outgrowth which could be completely rescued by ERK-inhibition (Fig. 4L). Increased ROCK-activity limits neurite outgrowth [34]. Thus, reduced neurite outgrowth would have been expected by enhanced ROCK activity. ROCK-inhibition, however, had no influence on neurite outgrowth in this SMA model suggesting that hyper-activation of the ERK-pathway overcompensates for enhanced ROCK-activity on the functional level. However, we could not link FGFR1-inhibition with increased neurite outgrowth, indicating enhanced ERK-activity independent of this particular receptor. As receptor tyrosine kinases often show redundancy in their signaling and function [35], it is likely possible that other receptors are upregulated instead of FGFR1.

Enhanced neurite outgrowth under SMN reduction has been described before. Primary motoneurons derived from SMA-mice and grown on neuromuscular laminn display prolonged axons [36]. However, for NSC34 cells this is a new finding and in contrast to previous reports [37,38]. Therefore, we included several controls and applied rigid statistics in our analyses. These results cannot be compared directly since both groups applied inducible systems for SMN knock-down instead of transient knock-down used in our study. Moreover, different growth conditions and neuronal differentiation protocols, such as 6 days of differentiation or re-seeding of differentiated cells, have been used in the other studies. Additionally, the above mentioned groups traced neurites by fluorescent staining which demands relatively low cell densities for discrimination of neurites. In contrast, we decided to seed the cells at high densities and trace single neurites by fluorescent proteins to assure comparability of biochemical and morphological analyses. Importantly, neurite outgrowth has

Fig. 6. Proposed model for bilateral ROCK–ERK crosstalk. ROCK and ERK pathways (boxes) show a mutual inhibitory function. In the monogenetic neurodegenerative disease spinal muscular atrophy (SMA), cellular concentration of the survival of motoneuron (SMN) protein is significantly decreased (red arrow). This ultimately leads to a disruption of the crosstalk. Communication between the pathways becomes limited to an unidirectional mode. In a motoneuron-like model of SMA, both ROCK and ERK pathways are hyper-activated (red arrows). By default, ERK and ROCK show antagonistic functions on regulation of neuronal differentiation. In the SMA model, however, the influence of the ERK pathway predominates leading to enhanced neurite outgrowth (red arrow).
been reported to be susceptible for changes in cell densities leading to different biological outcomes by the same treatment conditions \[39,40\]. In contrast to NSC34 cells, we could previously show a reduction of neurite outgrowth in a PC12 cell model for SMA, induced by widespread ROCK-pathway dysregulations \[9\]. SMN binds directly to the actin-binding protein profilin-2a (profa2). Under SMN knock-down conditions, a profa2 hyper-phosphorylation as well as a reduction of cofilin-phosphorylation has been demonstrated. Reduced SMN levels in SMA cause a profa2 release from its complex with SMN, thereby increasing its binding to ROCK \[10\] and inducing its hyper-phosphorylation \[9\]. In turn, the equilibrium activity of ROCK towards its other downstream targets becomes disturbed resulting in hypo-phosphorylation of cofilin and myosin light chain phosphatase \[9\]. Cofilin and profa2 are both involved in actin cytoskeleton and neurite outgrowth regulation. Interestingly, ROCK-inhibition fully rescues the neurite outgrowth defect emphasizing the importance of that pathway in PC12 cells \[9\]. However, this study shows that an SMN knock-down in PC12 cells does not result in dysregulation of the ERK-pathway (Fig. 2C, D). This is in accordance with the previously reported ROCK-dependancy of decreased neurite outgrowth \[9\]. Thus, PC12 and NSC34 cell-based models of SMA exhibit fundamental differences in processing signals controlling neurite outgrowth thereby displaying specific molecular profiles of ERK- and ROCK-pathways. This ultimately leads to opposite outcomes: NSC34 cells displayed enhanced neurite outgrowth while PC12 cells show a decrease in process elongation. NSC34 cells show motoneuron-like features expressing many respective markers and inducing acetylcholine receptor clusters on co-cultured primary myotubes \[22\]. PC12 cells originate from a rat tumor of the adrenal medulla and show sympathetic neuron-like features when differentiated with NGF \[41\]. Interestingly, no primary outgrowth defects in motoneurons have been reported for SMA-mice, while sympathetic neurons do show impairments. SMA-mice display reduced sympathetic innervation with fewer branches of the heart linked with bradyarrhythmia \[42\]. Supporting these observations, SMA-patients suffer from impaired autonomic nerve function \[43,44\]. With regard to the motoneuron phenotype, a major difference between mild and severe SMA-mice is their ability for re-innervation. In mild models, the phase of denervation is typically followed by a phase of terminal sprouting and re-innervation leading to enlarged motor units \[45\], a phenomenon also observed in milder affected patients \[46\]. This process does not occur in severe SMA, which could be considered as a neuronal outgrowth defect as well. Although ERK is in principle beneficial for neuronal survival and outgrowth \[16\], enhanced ERK-activity could be linked to disturbed axonal outgrowth. Motoneurons implanted in neural tubes of chick embryos fail in proper guidance when expressing constitutively active MAPK/ERK kinase 1 (MEK), which is an ERK upstream agonist \[47\]. Thus, a fine regulation of pathways controlling neurite outgrowth is needed in complex physiological contexts. Therefore, our molecular model of sustained dysregulated ROCK- and ERK-pathways (Fig. 6) is significant for dissecting the relevant patho-physiological pathways in SMA.

Importantly, treatment of SMA-mice with ROCK- as well as with ERK-inhibitors both led to enhanced survival and improved motoneuron phenotypes \[17–19\]. Since we demonstrated both, enhanced ERK- as well as ROCK-activity in motoneuron-like NSC34 cells, this strongly supports relevance of our results for a new therapeutic strategy. Treatment with ROCK-inhibitors Y27632 and Fasudil, respectively, improved survival and weight gain in type 2-like SMA mice \[18,19\]. Severely affected mice did not display any benefits for Fasudil \[18,19\]. Application of ERK-upstream inhibitor Selumetinib, however, led to a significant increase in lifespan and weight gain of severely affected mice \[17\]. Importantly, the authors reported an enhanced ERK-activity in SMA-mice spinal cords. ERK-inhibition induced a shift towards activated Akt-signaling thereby promoting SMN-expression. These expression changes were induced by enhanced binding of a transcriptional activator downstream of Akt competing with a transcriptional suppressor downstream of ERK. Thus, the authors proposed a mechanism in which enhanced ERK-activity amplifies SMN-reduction. ERK-inhibition improved the phenotype by shifting activity towards beneficial Akt signaling resulting in increased SMN expression \[17\]. Moreover, another putative mechanism for disadvantageous ERK-activation was proposed. Increased ERK-activity was found in SMA-patient derived astrocyte-like induced pluripotent stem cells (iPSCs). The authors suggested that this might lead to an enhanced secretion of pro-inflammatory cytokines inducing apoptosis in surrounding motoneurons \[48\].

Our findings argue for a third putative mechanism in which ERK-inhibition corrects for motoneuron-intrinsic ERK-hyperactivation thereby adjusting outgrowth-regulating pathways. The demonstration of a bilateral crosstalk hints for possible negative effects of those treatment strategies. As ERK-inhibition induces ROCK activation and vice versa, inhibition of just one of those pathways disadvantageously enhances activity of the respective crosstalk partner. Although ERK to ROCK crosstalk is impeded in our SMA-model system, the observed increase of SMN-expression in ERK-inhibitor treated mice \[17\] might abolish this effect. Thus, our crosstalk analysis reveals potential disadvantages of separately applied ROCK- and ERK-treatment strategies. A therapeutic strategy with both therapeutic compounds might therefore exert synergistic beneficial effects on SMA symptoms.

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Author contributions
N.H. performed and designed most experiments; I.S., S.R., N.B. and H.B. performed some experiments; P.C. mentored the project and designed experiments; N.H., C.G. and P.C. wrote the manuscript.

Appendix A. Supplementary data
Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cellsig.2013.11.027.

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