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SUMMARY

Neurotransmission defects and motoneuron degeneration are hallmarks of Spinal Muscular Atrophy, a monogenetic disease caused by the deficiency of the SMN protein. In the present study, we show that systemic application of R-Roscovitine - a Ca\textsubscript{v}2.1 / Ca\textsubscript{v}2.2 channel modifier and a Cyclin-dependent kinase 5 (Cdk-5) inhibitor - significantly improved survival of SMA mice. In addition, R-Roscovitine increased Ca\textsubscript{v}2.1 channel density and sizes of the motor end-plates. In vitro, R-Roscovitine restored axon lengths and growth cone sizes of Smn-deficient motoneurons corresponding to enhanced spontaneous Ca\textsuperscript{2+} influx and elevated Ca\textsubscript{v}2.2 channel cluster formations independent of its capability to inhibit Cdk-5. Acute application of R-Roscovitine at the neuromuscular junction significantly increased evoked neurotransmitter release, the frequency of spontaneous miniature potentials, and lowered the activation threshold of silent terminals. These data indicate that R-Roscovitine improves Ca\textsuperscript{2+} signaling and Ca\textsuperscript{2+} homeostasis in Smn-deficient motoneurons which is generally crucial for motoneuron differentiation, maturation, and function.
INTRODUCTION

Proximal spinal muscular atrophy (SMA) is the most common form of fatal motoneuron disease in childhood. SMA is caused by loss or compound heterozygous mutations within the survival motoneuron gene (SMN1) that results in deficiency of the SMN protein (Lefebvre, et al. 1997). Although genetically issued therapeutic strategies (Hua, et al. 2011; Naryshkin, et al. 2014; Ottesen 2017; Palacino, et al. 2015; Van Alstyne and Pellizzoni 2016) are under consideration, it is unknown how SMN deficiency dysregulates cellular mechanisms in motoneurons. A defective Ca\(^{2+}\) homeostasis was discussed in glial and neuronal cells in SMA animal models (Biondi, et al. 2010; Biondi, et al. 2008; Jablonka, et al. 2007; Lyon, et al. 2013; McGivern, et al. 2013; Riessland, et al. 2017; Ruiz, et al. 2010; Subramanian, et al. 2012). Embryonic motoneurons in primary cultures from SMA mouse models show reduced N-type (Ca\(_{v}\)2.2) voltage-gated calcium channel (VGCC) cumuli in their protrusions (Jablonka, et al. 2007) that corresponds to altered axonal extension on a synapse-specific laminin isoform (laminin-221) and on a Schwann Cell-specific isoform (laminin-111) (Jablonka, et al. 2007; Rossoll, et al. 2003). At the neuromuscular junction (NMJ), motor nerve terminals from SMA mice also display a reduction in P/Q-type (Ca\(_{v}\)2.1) VGCCs (Tejero, et al. 2016), that correlates with the neurotransmission deficit (Kong, et al. 2009; Ruiz, et al. 2010).

To test whether the re-balancing of the dysregulated Ca\(^{2+}\) homeostasis compensates for motoneuron defects, we used the small molecule Roscovitine. R- and S-Roscovitine are stereoisomers originally described as equally effective inhibitors of cyclin-dependent kinases (Cdk), in particular Cdk-5 (Meijer, et al. 1997; Meijer et al., 2016). In addition, R-Roscovitine slows the deactivation (closing) kinetics of Ca\(_{v}\)2.1 and Ca\(_{v}\)2.2 channels what prolongs the open-state of the channel and increases calcium influx (Yan, et al. 2002). In contrast, S-Roscovitine has no effect on the deactivation of the channels and even can inhibit Ca\(^{2+}\) influx (Buraei and Elmslie 2008). In vitro, R-Roscovitine increases Ca\(^{2+}\) influx and
transmitter release at presynaptic terminals of cultured hippocampal neurons independent from its Cdk-5 inhibitory effect (Buraei, et al. 2005; Yan, et al. 2002). In frog neuromuscular preparations, R-Roscovitine but not S-Roscovitine increases the number of acetylcholine quanta released during electrical stimulation (Cho and Meriney 2006).

Our data indicate that R-Roscovitine increases SMA mouse survival and beneficially supports motoneuron differentiation, as well as synapse maintenance in the spinal cord and counteracts morphological defects of the neuromuscular junction (NMJ) in a SMA mouse model (Smn<sup>−/−</sup>;SMN2;SMN<sub>∆7</sub>). Finally, acute R-Roscovitine application induces Ca<sup>2+</sup> transients <em>in vitro</em> and increases quantal content <em>ex vivo</em>. We conclude that these effects are primarily mediated through the capability of R-Roscovitine to enhance Ca<sup>2+</sup> currents.
RESULTS

Increased mean live span of SMA mouse models upon systemic R-Roscovitine treatment.

We used different severe SMN\Delta7 mouse models (groups 1 & 2, Figure 1A) to investigate the effect of R-Roscovitine on their survival. To discern whether the impact of R-Roscovitine is due to its inhibitory effect on Cdk-5, we used S-Roscovitine as a control, which inhibits Cdk-5 as well as R-Roscovitine but does not increase Ca\^{2+} influx. Pregnant heterozygous SMA mice were daily injected with R- or S-Roscovitine (2 mg/kg; see also SI_Transparent Methods). In group 1, the mean survival of the homozygous SMA mice (Smn\(^{-/-}\);SMN2tg/x;SMN\Delta7tg/x) treated with R-Roscovitine significantly increased (13.4±6.2 days) in comparison to untreated SMA mice (4.9±2.0 days) (Figure 1B, C). Additional postnatal application of R-Roscovitine did not prolong survival of SMA mice (13.9±5.5 days) (Figure 1B, C). To verify whether a consistent double transgenic (SMN2tg/tg;SMN\Delta7tg/tg) background increases the mean survival after R-Roscovitine application, pregnant Smn\(^{+-}\);SMN2tg/tg;SMN\Delta7tg/tg mice were injected. The SMN2tg/tg;SMN\Delta7tg/tg background (group 2) increased the mean survival rate up to approximately 14 days (14.0±1.1 days) (Figure 1B blue curve; Figure 1C blue bar) comparable to previous reports (Le, et al. 2005). R-Roscovitine application again elevated the mean survival significantly up to 19 days (18.9±1.7 days) (Figure 1B, pink curve; Figure 1C pink bar). However, the maximum value of 23 days upon R-Roscovitine application to group 1 mice (Figure 1B, green curve) was not exceeded by the double transgenic SMN2/\Delta7-background (22 days) (Figure 1B, pink curve). No significant weight gain (until P9), but a non-significant delayed weight loss up to P12 had been observed following prenatal R-Roscovitine application in SMA mice from group 2 (Figure S1A). However, a slight but non-significant effect on the righting reflex between P3 and P6 was detectable (Figure S1B).

Prenatal injection of S-Roscovitine had no beneficial effect on the survival of SMA mice (5.7±2.3 days) (Figure 1B grey curve, Figure 1C grey bar). To test if R-Roscovitine
compensates for reduced survival of SMA mice when first clinical symptoms already
appeared, Smn-deficient mouse pups from group 1 received a postnatal injection at P2 with R-
Roscovitine (1.5 mg/kg) reaching a blood serum concentration of about 50-100 µM (see also
SI_Transparent Methods). Application of R-Roscovitine significantly extended the mean life
span to 9.3±2.7 days, compared to Smn-deficient pups treated with PBS (3.8±1.8 days) or
DMSO (5.8±1.5 days) (Figure 1D, E). Representative examples of postnatal treated and non-
treated mice from group 1 are depicted in Figure 1F-I.

**Prenatal treatment with R-Roscovitine decreases loss of spinal motoneurons, increases
the number of excitatory somatodendritic inputs on motoneurons, and beneficially
affects Ca\textsubscript{v}2.1 channel cluster formation in SMN\textDelta7 mice.**

In order to analyse the cellular effects of R-Roscovitine on motoneuron loss, the number of
excitatory somatodendritic inputs, the area of neuromuscular junctions, Ca\textsubscript{v}2.1 cluster
formation as well as muscle fiber calibre were compared between control versus SMA mice
(group 2). To check whether the prenatal treatment with R-Roscovitine changed the number
of spinal motoneurons in SMA mice, acetyl-transferase (ChAT) positive cells were labeled,
and their number estimated at the upper lumbar regions (L1-L2), which are particularly
vulnerable in SMA (Mentis, et al. 2011). Figure 2A shows an example of the distribution of
labeled neurons and the mean number of motoneurons estimated in non-treated and prenatally
treated control and SMN\textDelta7 mice. In the absence of the drug, mutants showed a significant
reduction by 40 % with respect to their control littermates. However, in treated mutants the
number of motoneurons was not significantly different from their controls (Figure 2A). We
next checked whether the number of excitatory somatodendritic inputs on motoneurons was
modified by the drug treatment. We found no significant differences in the density or the
number of vGluT2 positive inputs per µm\textsuperscript{2} at the soma between mutants and their littermate
controls in the group treated with R-Roscovitine, contrarily to that found in the non-treated
group (Figure 2B). To test whether R-Roscovitine had an effect on the pre- and postsynaptic side of the NMJ, we quantified the postsynaptic area (Figure 2C), and determined the Ca$_{v}$2.1 cluster formation in the presynaptic compartment (Figure 2D) of the transversus abdominis (TVA), one of the most affected muscles in the disease model (Tejero, et al. 2016; Torres-Benito, et al. 2011). While in non-treated mice the endplate surface area was reduced significantly in mutants in comparison to littermate controls, no differences were found between the two genotypes when mice were treated with R-Roscovitine (Figure 2C). Additional effects were observed after antibody-staining against the P/Q-type VGCC (Ca$_{v}$2.1). R-Roscovitine beneficially affected Ca$_{v}$2.1 cluster formations in mutant NMJs indicated by the ratio between P/Q area and BTX area (Figure 2D upper and lower panel).

Finally, we investigated muscle fiber area and perimeter of the TVA. Contrarily, no improvement in myofiber surface area or perimeter was found following R-Roscovitine treatment (Figure 2E). To figure out whether muscle innervation is influenced by R-Roscovitine treatment we used the flexor digitorum brevis (FDB3) muscle, which has been described to be one of the most denervated in Smn-deficient mice (Ling, et al. 2012). Surprisingly, we found a low number of unoccupied endplates in both genotypes, and no significant changes in R-Roscovitine-treated mice compared with untreated mice (Figure S1C).

**R-Roscovitine increases calcium signaling of primary cultured SMA motoneurons.**

The *in vivo* results raised the possibility that the delay in motoneuron loss and the larger P/Q-VGCC surface area at motor nerve terminals were, in part, due to the capability of R-Roscovitine to slow down the deactivation kinetics of Ca$_{v}$2.2/2.1 channels and, therefore, to increase cytosolic Ca$^{2+}$ levels. Thus, we analysed acute and long-term effects of the drug on axons and axonal nerve endings of isolated embryonic motoneurons from Smn-deficient mouse embryos. Since SMA motoneurons cultured on the β2 chain-containing laminin
isoform (laminin-221) display a reduction in spontaneous spike-like Ca$^{2+}$ transients and reduced Ca$_{v}$2.2 cluster formations at the growth cone (Jablonka, et al. 2007), we examined whether R-Roscovitine modulates Ca$^{2+}$ transients in motoneurons. We started our approach with an acute application of 5 µM R- as well as S-Roscovitine, given that primary cultured motoneurons are very vulnerable and that in cultured hippocampal neurons acute application of 10 µM R-Roscovitine effectively increases synaptic potentials (Tomizawa, et al. 2002). In control motoneurons at 5 days in vitro (DIV 5) the acute application of 5 µM R-Roscovitine, but not S-Roscovitine, led to an immediate and continuous induction of Ca$^{2+}$ transients both in soma and growth cone (Figure 3A), while acute application of 0.5 µM R-Roscovitine had no effect (Figure S2A). Next, we wanted to know whether the Ca$^{2+}$ transients evoked by acute R-Roscovitine application might also cause a continuous/general increase of spontaneous Ca$^{2+}$ transients in Smn-deficient motoneurons during chronic exposure to the drug over 5 days.

Thus, we investigated the long-term effects of both drugs on motoneurons in vitro. Since R- or S-Roscovitine at high concentrations (>1 µM) diminished survival in dissociated motoneurons over 5 days in culture (Figure S3A), all further long-term in vitro experiments were performed with an end concentration of 0.5 µM R- or S-Roscovitine. Chronic R-Roscovitine treatment for 5 days compensated the reduced frequency of spontaneous spike-like Ca$^{2+}$ transients at Smn-deficient distal axons (Figure 3B, right and middle panel). Single values per neuron are depicted in figure 3B, left panel. This effect was blocked when 0.3 µM ω-conotoxin (ω-CTX-MVIIC), a Ca$_{v}$2.2/2.1 inhibitor, was present during the cell culture period (Figure S2B). Nevertheless, permanent application of 0.5 µM S-Roscovitine for 5 days to Smn-deficient motoneurons resulted in a moderate - but not significant - increase in the frequency of spontaneous spike-like transients (Figure 3B, left, right and middle panel). To rule out VGCC affecting attitudes of Cdk-5, motoneurons from Cdk-5 knockout mouse embryos were cultured with and without 0.5 µM R-Roscovitine over 5 days and processed for calcium imaging studies at DIV 5. Cdk-5-deficiency had no effect on spontaneous Ca$^{2+}$
transients (Figure S2C). Taking together, these observations are compatible with the calcium channel modulating properties of R-Roscovitine through its ability to slow the closing-time of Ca_{v}2.1 and Ca_{v}2.2 channels. Furthermore, the potential inhibition of Cdk-5 by 0.5 µM R-Roscovitine did not impact its capability to rescue altered excitability in axon terminals of primary cultured Smn-deficient motoneurons.

**Chronic application of R-Roscovitine improves differentiation of SMA motoneurons in culture.**

In order to figure out whether the increase of spontaneous Ca^{2+} transients might cause long-term support of cellular differentiation of Smn-deficient motoneurons, we performed a morphological analysis. Given that Smn-deficient motoneurons in culture display smaller growth cone sizes (Jablonka, et al. 2007), we checked the ability of R-Roscovitine (0.01-0.5 µM, for 5 days) to compensate it. We found that the effect of R-Roscovitine acts in a dose-dependent manner, with 0.5 µM being required for full compensation (Figure 4A-D). Strikingly, mean signal intensity of the calcium channel “clusters” measured by immunofluorescence was also restored to control level upon permanent R-Roscovitine treatment over 5 days (Figure 4A-C, E). These effects of R-Roscovitine were independent of Smn protein levels as the total transcript and protein amount was unaffected (Figure S3B, C).

In addition, reduced spontaneous Ca^{2+} transients in Smn-deficient motoneurons and ω-CTX-MVIIC-treated control cells are associated with altered axon elongation on different laminin isoforms (Jablonka, et al. 2007). In the presence of 0.5 µM R-Roscovitine, however, axon lengths of Smn-deficient motoneurons, both on Schwann cell-specific laminin (laminin-111) and in the synapse-specific laminin (laminin-221/211) reached control values on day 7 (DIV 7) (Figure 4F). The presence of 0.3 µM ω-CTX-MVIIC over DIV 7 increased the length of control axons to mutant level and prevented the R-Roscovitine induced rescue in mutant cells on both laminin isoforms (Figure 4F). S-Roscovitine did not affect axon elongation on
laminin-111 or laminin-221/211 significantly (Figure 4G), which supports the notion that R-Roscovitine modulates motoneuron differentiation via Ca\(_{\text{v2}}\) channels. To further test this hypothesis, we compared axon lengths of Smn-deficient motoneurons following treatment with R-, S-Roscovitine, and GV-58. GV-58, an N- and P/Q-type VGCCs agonist with a 20-fold lower cyclin-dependent kinase-antagonist-activity (Liang, et al. 2012), has been already investigated in a mouse model for Lambert-Eaton Syndrome (Tarr, et al. 2013). GV-58 application for 7 days adjusted axon lengths of Smn-deficient motoneurons to wild type level on laminin-221/211, like R-Roscovitine but not S-Roscovitine (Figure 4H). Finally, we also checked whether 0.5 µM R-Roscovitine has any effect on the Cdk-5 substrate Pak1 or the p35/25 activators and found no changes in Western blot measurements (Figure S4A-C).

**Gene expression of Ca\(_{\text{v2.2/2.1}}\) channels is not up- or downregulated in cultured SMA motoneurons following R-Roscovitine treatment.**

Previous reports have shown altered gene expression under Smn-deficiency (Doktor, et al. 2017; Fletcher, et al. 2017; Jablonka and Sendtner 2017; Saal, et al. 2014). This poses the question of whether the beneficial effect(s) on VGCCs via R-Roscovitine are due to upregulated gene expression of Ca\(_{\text{v2.2/2.1}}\) channels. To explore this, we collected the RNA for high-throughput sequencing (RNASeq) from primary Smn-deficient and control motoneurons cultured on laminin-111 and treated with and without R-Roscovitine, respectively (Figure 5, Table S1-S7). Differential expression analysis revealed 2404 transcripts that were significantly (p<0.05) altered in untreated \(\text{Smn}^{-/-};\text{SMN2}\) compared to untreated \(\text{Smn}^{+/+};\text{SMN2}\) motoneurons (1011 up- and 1393 downregulated transcripts) (Figure 5A, Table S1). The transcript level of \(\text{SMN2}\) is not affected by R-Roscovitine (Figure S3B). Addition of R-Roscovitine induced significant alterations of 612 transcripts (464 up- and 148 downregulated transcripts) in \(\text{Smn}^{-/-};\text{SMN2}\) motoneurons and of 898 transcripts (582 up- and 316 downregulated transcripts) in \(\text{Smn}^{+/+};\text{SMN2}\) motoneurons (Figure 5A, Tables S2 and S3).
Strikingly, of the 612 transcripts whose levels were changed by R-Roscovitine in Smn-deficient motoneurons 357 were also significantly altered between $Smn^{+/+};SMN2$ and $Smn^{+/+};SMN2$ motoneurons (Figure 5B, Table S4). The magnitude of change for these 357 transcripts was strongly negatively correlated with a Pearson correlation coefficient of -0.87 (Figure 5C). As a result, we detected 305 transcripts that were downregulated in $Smn^{-/-};SMN2$ compared to $Smn^{+/+};SMN2$ motoneurons and upregulated by R-Roscovitine treatment in $Smn^{-/-};SMN2$ motoneurons (Figure 5C, Table S5). Subunits of Ca$\text{v}_{2.2}$, as well as, Ca$\text{v}_{2.1}$ were not among this set of transcripts (Table S5). Gene Ontology (GO) term analysis of these 305 transcripts revealed enrichment of transcripts encoding proteins with functions in neuronal differentiation, neurotransmitter transport and location at the synapse (Figure 5D, Table S6). We also detected 40 transcripts that were upregulated in $Smn^{-/-};SMN2$ compared to $Smn^{+/+};SMN2$ motoneurons and downregulated by R-Roscovitine treatment in $Smn^{-/-};SMN2$ motoneurons (Figure 5C, Table S7). Taken together, R-Roscovitine treatment partially rescued the transcriptome perturbations induced by reduced $SMN$ expression, although no effect on the expression of Ca$\text{v}_{2.2}$, as well as Ca$\text{v}_{2.1}$ channels was detectable.

**Acute exposition to R-Roscovitine increases neurotransmitter release in motor nerve terminals from SMA and control mice.**

Our data suggest that the cell-autonomous and excitability-associated defects of cellular differentiation in primary Smn-deficient motoneurons (Figures 3 and 4) and of the neuromuscular system in SMA mice (Figure 1 and 2) can be improved by the modulatory effect of R-Roscovitine on VGCCs. Thus, we decided to transfer our studies on *ex vivo* neuromuscular preparations from SMNΔ7 (group 2) mice. We investigated if R-Roscovitine was able to increase neurotransmitter release at the NMJ by performing intracellular recordings of postsynaptic potentials in the presence and the absence of the drug. The experiments were performed in the TVA muscle of Smn-deficient and littermate control mice.
at P9-P11, a stage at which neurotransmission is highly affected (Kariya, et al. 2008; Kong, et al. 2009; Ruiz, et al. 2010; Tejero, et al. 2016; Torres-Benito, et al. 2011). Following the calculated concentrations achieved by subcutaneous application of R-Roscovitine in pregnant mice and postnatal mouse pups (see SI_Transparent Methods), in each experiment, a number of fibers were first recorded in the absence of the drug (control solution) and then in the presence of R-Roscovitine (10, 50, 100 µM) after 20-30 min of incubation. 100 µM R-Roscovitine did not modify the amplitude of spontaneous mEPPs, neither in control nor SMA terminals (Figure 6A), but significantly increased the mEPP frequency in both genotypes (Figure 6B). The EPP amplitudes greatly increased in control (~280%) and SMA (~295%) terminals in the presence of 100 µM R-Roscovitine. (Figure 6C, E). Quantal content (QC), the ratio between mean EPP and mean mEPP amplitudes, were similarly elevated in the presence of the drug both in control and SMA mice (Figure 6D, F). In contrast, 10 µM R-Roscovitine had no effect on mEPP and EPP amplitudes or quantal content in control or SMA nerve terminals (Figure S2D-F). Although 50 µM showed no significant effect, it tended to increase EPP amplitude and quantal content. This is in line with the effective dose to increase neurotransmitter release at the adult frog NMJ (Cho and Meriney 2006). S-Roscovitine and Olomoucine, another structurally related cyclin-dependent kinase inhibitor, were not able to increase neurotransmitter release, similarly to what it has been described before at the frog NMJ (Cho and Merimey, 2006). In fact, S-Roscovitine had a significant inhibitory effect on neurotransmission, which was partially reversible (Figure 6G, H), while Olomoucine (100 µM) had no effect at all (QC: 6.02 ± 0.36 in the absence, and 5.72 ± 0.21 in the presence of the drug, 6 fibers in each condition).
Acute application of R-Roscovitine increases the probability of neurotransmitter release and unmasks silenced terminals in SMA and control nerve terminals.

To explore whether R-Roscovitine changes the probability of vesicle release, we measured the synaptic responses to two consecutive stimuli (pair pulse protocol, interstimulus interval 50 ms). In the absence of R-Roscovitine, the mean amplitude of the second EPP was larger than that of the first, indicating an increase in release probability between stimuli (short-term facilitation) in control and SMA terminals (Figure 7A, upper traces). In the presence of 100 µM R-Roscovitine, the synaptic facilitation significantly disappeared in both control and SMA mouse (Figure 7A lower panels, and 7B), suggesting a general increase in release probability.

Remarkably, the acute application of R-Roscovitine also significantly increased the number of muscle fibers that responded with double EPPs to a single nerve stimulus (Figure 7C, D). Double EPPs are a typical sign of multi-innervation, and the different timings of the peaks (Figure 7C) result from the distinct conduction velocities of each active axon terminal. Double EPPs are often recorded perinatally but become less frequent by two weeks of age as most muscle fibers become mono-innervated (Sanes and Lichtman 1999). At P9-11, only a small percentage of the entire recorded fibers in the control solution presented double EPPs (Figure 7E, control ~ 7%; mutant: ~22%). By R-Roscovitine exposure, the fraction of recorded fibers with double EPPs increased in control and SMA mutants (Figure 7E, control: ~ 48%; mutant: ~67%).

R-Roscovitine effect on neurotransmitter release requires the activation of P/Q-type voltage-gated calcium channels.

We next explored whether the R-Roscovitine effect on the NMJ required the activation of P/Q-type channels (CaV2.1), the main VGCC at the age at which the recordings were made (Rosato Siri and Uchitel 1999; Santafe, et al. 2001). For this purpose, we used 200 nM ω-
Agatoxin IVA (ω-Aga.) as a specific blocker of P/Q-type channels (Rosato Siri and Uchitel 1999), and found that the mean amplitude of EPPs was reduced by ~ 99.2% (Figure 8A, left bar graph) and the quantal content by ~ 99.1% (Figure 8A, right bar graph). Adding 100 µM R-Roscovitine to terminals with ω- Agatoxin IVA still being present did not increase the EPP amplitude or the quantal content (Figure 8A). Accordingly, in control mouse terminals where exposition to R-Roscovitine resulted in ~84% mean increase in neurotransmitter release, subsequent application of ω- Agatoxin IVA completely blocked the evoked response (Figure 8B). In Smn-deficient mice, similar effects occurred, ω- Agatoxin IVA completely blocked neurotransmitter release after R-Roscovitine had increased EPP amplitude and quantal content (Figure 8B). These results indicate that R-Roscovitine was not able to affect motor terminal release when P/Q-type VGCCs were blocked with ω- Agatoxin IVA, suggesting that R-Roscovitine does not increase release through the activation of a different calcium source.
DISCUSSION

Here, we demonstrated that in vivo treatment of SMNΔ7 mice with R-Roscovitine increases survival, beneficially affects formation of Ca_{v}2.1 channel clusters at the motor nerve terminal, increases the size of the NMJ, and tends to counteract loss of motoneuron and excitatory somatodendritic inputs on spinal motoneurons. Improved preservation of the neuromuscular system corresponds to increased frequency of spontaneous calcium transients, ameliorated axonal length defects, increased growth cone sizes, and modifications of some of the transcriptome perturbations in Smn-deficient motoneurons. Acute application of R-Roscovitine increases neurotransmitter release in control and SMA motor nerve terminals and favors the recruitment of silenced terminals. Together, these data reveals that R-Roscovitine is a substance that in general supports motoneuron differentiation, function, and maintenance.

**R-Roscovitine treatment prolongs the survival of SMA mice.**

R-Roscovitine improves cellular differentiation and maturation of SMA motoneurons in vitro and increases neurotransmission at the NMJ in ex vivo neuromuscular preparations. Based on our data, the possibility that R-Roscovitine mediates its beneficial effects on the neuromuscular system via direct modulation of the Ca_{v}2.1 and Ca_{v}2.2 channels are comprehensible and convincing. However, we cannot discard additional mechanisms, especially regarding the improvement of SMA mouse survival. R-Roscovitine binds with high affinity to Cdk-1, 2, 5, 7, 9, and a few kinases such as CaM kinase 2 (CaMK2A), extracellular signal-regulated kinases 1 and 2 (ERK1/2), and PDXK Pyridoxal kinase) (Dhorajiya et al., 2012). Inhibitors of cyclin-dependent kinases impact cell cycle mechanisms and are used for anti-neoplastic therapies (Bruyere and Meijer 2013). Furthermore, targeting CaMK2, Erk1, and Erk2 by R-Roscovitine or its analogs might influence motoneuron differentiation/maturation. Additionally, inhibition of Cdk-5/p35 by Roscovitine could partially contribute to enhanced activity of the Ca_{v} channels by increasing their interaction...
with some SNARE proteins (Tomizawa, et al. 2002). Finally, it should be mentioned that COOH-R-Roscovitine, the main metabolite of R-Roscovitine, although it has less Cdk- and kinase-inhibition-activity (Nutley, et al. 2005), may have adverse or beneficial side effects that need to be illustrated separately in future long-term approaches on SMA animal models. In principle, the complete survival phenotype is not necessarily explained by the herein studied cellular/molecular mechanisms of R-Roscovitine action, and additional work would be needed to understand this phenotype.

**Long-term exposition to R-Roscovitine improves differentiation of primary cultured Smm-deficient motoneurons.**

Our systematic R- and S-Roscovitine concentration gradient study on the survival of motoneurons (Figure S3A) discovered that the optimal dose for *in vitro* chronic treatment with R-Roscovitine is 0.5 µM, which is not toxic but still compensates morphological and functional abnormalities. The parameters used to assess cellular differentiation were growth cone sizes (Figure 4A-D) and axon lengths (Figure 4F-H) on one side, and calcium channel density at SMA growth cones (Figure 4E) on the other. The observed improvements correlate with the marked enhanced spontaneous Ca\(^{2+}\) influx in embryonic SMA motoneurons (*Smm^-/- ;SMN2*) chronically treated with R-Roscovitine (Figure 3B). We found no effects on wild-type motoneurons, probably because their cellular differentiation already proceeded correctly and thus is not further improvable by calcium increment. Upon S-Roscovitine treatment (0.5 µM), which inhibits Cdk-1, Cdk-2, and Cdk-5 enzymatic activity with a similar IC\(_{50}\) as R-Roscovitine, we observed a slight but not significant increase of spontaneous Ca\(^{2+}\) transients after long-term application (Figure 3B) without compensation of morphological and functional abnormalities in Smm-deficient motoneurons (Figure 4G). These results, together with the lack of effect of S-Roscovitine on mouse survival (Figure 1) and neurotransmitter release (Figure 6) imply that the inhibition of cyclin-dependent kinases by Roscovitine - or by
its metabolites - is not enough to improve the SMA phenotype and argues for the importance of R-Roscovitine on Ca\(^{2+}\) influx, probably by its direct action on calcium channels. This idea is further supported by two of our observations. Firstly, we discovered, that GV-58, another Ca\(_{v}2.1\) and Ca\(_{v}2.2\) agonist with a 20-fold lower Cdk-1, Cdk-2, and Cdk-5 activity than R-Roscovitine (Tarr, et al. 2013), leads to a significantly reduced axon elongation in Smn-deficient motoneurons on laminin-221/211, comparable to R-Roscovitine (Figure 4H). And, second, Cdk-5 depletion in primary cultured motoneurons does not impact spontaneous Ca\(^{2+}\) influx in long-term studies (Figure S2C), arguing for an R-Roscovitine mechanism being primarily focused on cellular excitability mediated through Ca\(_{v}2.1\) as well as Ca\(_{v}2.2\).

However, Roscovitine has been shown to affect other ion conductances, for example, it can inhibit L-type VGCCs and several types of potassium channels (Buraei, et al. 2007; Yarotskyy, et al. 2010). Indeed, some L-type antagonists, such Nifedipine, increases neurotransmitter release at the NMJ (Balezina, et al. 2007; Rosato Siri and Uchitel 1999; Sugiura and Ko 1997). Nevertheless, the fact that both R- and S-Roscovitine increase the inactivation of L-type channels (Yarotskyy, et al. 2010) makes less likely the hypothesis that the effects on EPP amplitude and quantal content in our system correspond to its effect on L-type VGCCs given the lack of positive modulation by S-Roscovitine in our experiments. On the other side, potassium channel blockers enhance secretion by broadening the action potential what results in a larger Ca\(^{2+}\) influx (Cho and Meriney, 2006). Interestingly, 4-Aminopyridine (4-AP), an efficient K\(_{v}\) channel blocker, can directly target presynaptic voltage-gated Ca\(^{2+}\) channels independent of its effect on K\(_{v}\) channels (Wu, et al. 2009), what would lead to the potentiation of neurotransmitter release by a double mechanism. All these possibilities need to be verified in the future by additional work.
R-Roscovitine does not affect Ca\textsubscript{v}2.2/2.1 gene-expression in Smn-deficient motoneurons.

Smn-deficiency affects transcript levels in various cellular systems and in vivo (Doktor, et al. 2017; Fischer, et al. 1997; Fletcher, et al. 2017; Imlach, et al. 2012; Li, et al. 2014; Liu, et al. 1997; Lotti, et al. 2012; Pellizzoni, et al. 1998; Saal, et al. 2014; See, et al. 2014; Wishart, et al. 2014; Zhang, et al. 2008). Thus, to investigate the eventuality that R-Roscovitine somehow alters cellular transcription levels of VGCCs, an RNASeq approach was performed (Figure 5). Interestingly, 305 transcripts are up-regulated by R-Roscovitine in Smn-deficient motoneurons, not including those of N-type, nor P/Q-type calcium channels (Table S5+6).

It is still an open question, why reduced Ca\textsubscript{v}2.2 cluster formations take place in Smn-deficient motoneurons. The auxiliary subunit \(\alpha_2\delta_2\) (Cacna2d2) of Ca\textsubscript{v}2 channels is one candidate (categorized in the “plasma membrane” Table S6). Cacna2d2 is one of four subunits \(\alpha_2\delta_1-4\) (Cacna2d1-4) that are loosely associated with VGCC-complexes, and modulate trafficking of Ca\textsubscript{v}2.1/2 (Dolphin 2012; Hoppa, et al. 2012). There is growing evidence that these auxiliary subunits may also have roles in the nervous system not directly linked to calcium channel function. In \textit{D. melanogaster} embryos, the \(\alpha_2\delta_3\) subunit plays a role in synaptogenesis independent of its association with calcium channels (Kurshan, et al. 2009). Other studies describe \(\alpha_2\delta_2\) as a developmental switch that regulates axon growth and regeneration, as \(\alpha_2\delta_2\) gene deletion or silencing promotes axon growth in vitro (Tedeschi, et al. 2016). In vivo, pharmacological blockade of \(\alpha_2\delta_2\) through Pregabalin administration enhances axon regeneration in adult mice after spinal cord injury (Tedeschi, et al. 2016). Therefore, it remains an open issue whether altered gene-expression of these auxiliary VGCC-subunits modifies Ca\textsubscript{v}2.1/2.2-cluster formations and in turn, affects neurotransmission in SMA mice.

SV2A and SV2C (Table S5) are two other candidates related to the regulation of the synaptic vesicles readily releasable pool size and the SNARE complex assembly. Tejero et al. (2016) could already show that the signal intensity of all three SV2 paralogs (A, B, & C) is significantly reduced in motor nerve terminals of skeletal muscles of SMA mice. Apart from
Smn-deficiency, impaired BDNF/trkB signaling causes a calcium-related phenotype in growth cones (Dombert, et al. 2017) similar to that observed in SMA motoneurons (Jablonka, et al. 2007) what opens the view on more complex pathways probably affected by Smn-deficiency leading to dysregulated motoneuron differentiation. Based on these data, we conclude that long-term application of R-Roscovitine might have effects on gene expression affected by Smn-deficiency. For a final conclusion that up- or downregulated cellular targets by R-Roscovitine have an impact on the SMA phenotype, additional experiments with relevant knockout or transgenic animal models are required.

**Acute application of R-Roscovitine improves synaptic transmission at the NMJ.**

In our *in vitro* assays, acute application of 5 µM R-Roscovitine increased Ca$^{2+}$ transients frequency (Figure 3), a concentration in line with experiments on cultured hippocampal neurons (Tomizawa, et al. 2002). However, postnatal motor nerve terminals required 100 µM R-Roscovitine to increase neurotransmitter release (Figure 6-8), in line with experiments in frog NMJs (Cho and Meriney 2006). The concentration difference among preparations could be partially explained by their respective state of development - EC$_{50}$ for Ca$_v$2.2 channels, predominant in cultured motoneurons, is about four-fold lower than for Ca$_v$2.1 channels, the most abundant at the NMJ (Buraei, et al. 2007; Tarr, et al. 2013) - and to the complexity of the postnatal motor nerve terminal architecture, although additional factors cannot be ruled out.

At postnatal stages, when SMN$\Delta$7 mice are highly affected, synaptic transmission is positively modulated by R-Roscovitine. This effect seems to be presynaptic as there was a reduction of facilitation (Figure 7A, B), indicative of an increase in vesicle release probability. Previous works also demonstrated that R-Roscovitine increases neurotransmitter release at the NMJ (Cho and Merney, 2006) and central synapses (Kim and Ryan 2010; Kim and Ryan 2013; Monaco and Vallano 2005; Tomizawa, et al. 2002; Yan, et al. 2002). This
effect has been shown to be mediated by modulation of VGCCs as R-Roscovitine slows the
deactivation kinetics of N- and P/Q-type calcium channels (Buraei, et al. 2005; Buraei and
Elmslie 2008; Buraei, et al. 2007; Yan, et al. 2002). The agonistic effect of R-Roscovitine is
Cdk-independent since intracellularly applied R-Roscovitine failed to affect Ca\textsubscript{v}2.2 channels
(i.e., to slow deactivation), in contraposition to its extracellular application (Buraei and
Elmslie 2008). At mouse motor nerve terminals, P/Q-type (Ca\textsubscript{v}2.1) channels are the main
channel involved in exocytosis (Katz, et al. 1997; Rosato Siri and Uchitel 1999; Santafe, et al.
2001; Tejero, et al. 2016). By using a specific toxin, we were able to show that R-Roscovitine
effects require the activation of P/Q-type calcium channels as ω-Agatoxin IVA totally
abolished neurotransmission. R-Roscovitine may directly interact on the calcium channel
(Yan, et al. 2002; Cho and Meriney, 2006; Tarr et al., 2013) or through the inhibition of Cdk-
5. The activation of Cdk-5 phosphorylates Ca\textsubscript{v}2.1 channels which decrease its interaction with
SNARE proteins and depresses neurotransmission (Tomizawa et al., 2002). However, we
found that S-Roscovitine decreased secretion, and Olomoucine had no effect at all, which
suggests that the second mechanism is less efficient in our conditions. We found that R-
Roscovitine was also able to wake up motor nerve terminals, which are silenced under control
conditions (Figure 7D). At the age our recordings were made (P 9-11), muscle fibers were in
the process of changing from multi- to mono-innervation (Murthy, et al. 2009; Ruiz, et al.
2010). It is not well known, however, how this process is regulated. One possibility we favor
is by shutting down the calcium channels implicated in neurotransmitter release (Miyazaki, et
al. 2004). The fast agonistic effect via reduced Ca\textsubscript{v}2.1 channel closing time favors R-
Roscovitine as a drug able to support neurotransmission rapidly in SMA.

In summary, our results show that R-Roscovitine in SMA regulates/modulates calcium influx,
motoneuron differentiation, enhances neurotransmitter release, and impacts gene expression.
While the data indicate that R-Roscovitine may exert many of the observed in vitro and ex

*vivo* effects by modulating Ca\(_{\text{x}2.1}\) and Ca\(_{\text{x}2.2}\) channels, we cannot exclude the participation of other targets as well, and thus additional experiments such as genetic manipulation of the calcium channels are needed. Moreover, the observed benefit on survival is not necessarily explained by the described calcium enhancement and will require additional work directed to identify other potential mechanisms. Everything considered, R-Roscovitine might support the current SMA therapies with antisense oligonucleotides (ASOs) (Ottesen 2017) or adenoviral SMN-gene transfer (Mendell, et al. 2017) in particular at disease onset - over a defined period - to support neurotransmission in a fast manner before SMN upregulation gives rise to its beneficial effects. Beyond this, R-Roscovitine could be useful in all motoneuron diseases with defects in Ca\(^{2+}\) homeostasis.
Limitations of the study

The beneficial effects of R-Roscovitine on the survival of SMA mice is not entirely explained by its cellular/molecular mechanisms, and further work is needed to understand this phenotype. Especially in the case of the voltage-gated calcium channels, knockout or knockdown approaches will narrow down the specific effects of R-Roscovitine on Ca\(_{2.2}\) and Ca\(_{2.1}\). Also the transcriptome-affecting effects of R-Roscovitine should be taken into account in further therapeutic applications.
METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental information includes Transparent Methods, References, Figures S1-S4, Tables S1-S7.

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AUTHOR CONTRIBUTIONS

Conceptualization and Project Administration, S.J. and L.T; Methodology, S.J., L.T., R.B., S.A., S.B., R.T.; Investigation, R.T., S.B., J.O., J.F–E., L.H., B.D., H.D., J.D.C., L.T.B., L.S.B., R.B.; Resources, S.J., L.T., R.B., S.A., M.B.; Formal Analysis, R.T., S.B., J.O., J.F–E., L.T.B., S.A., M.B.; Writing – Original Draft, S.J., L.T., S.B., R.T.; Funding Acquisition, S.J. and L.T.; Supervision, S.J. and L.T.

DECLARATION OF INTERESTS

The authors declare no competing interests.
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FIGURE LEGENDS

Figure 1: Prolonged live span of SMA mice under systemic R-Roscovitine treatment.

(A) Genotype of Smn-deficient mice used in the study. The phenotype in group 1 is more severe than in group 2. (B) Survival curve and (C) mean survival rate of SMA mice in group 1: untreated (black), prenatally treated with R- or S-Roscovitine (green and grey, respectively), and pre- and postnatally treated SMA mice (purple); and in group 2: untreated (blue) and R-Roscovitine (pink) (**p<0.01; ***p<0.001, ANOVA and U-Mann Whitney). (D) Survival curve and (E) mean survival rate of group 1 postnatally treated SMA mice with DMSO (orange), PBS (yellow) and R-Roscovitine (red) (**p<0.001, ANOVA). (F-I) Representative images for each example from postnatal application depicted at days 2, 5, 10 and 12. Bars represent mean±SD; n defines the number of mice; n.s.: no significance.

Figure 2: Prenatal treatment with R-Roscovitine reduces spinal motoneuron loss, ameliorates the decrease of excitatory synaptic inputs, and increases endplate surface area and Ca,2.1 accumulation area, but not muscle fiber size in SMNΔ7 mice.

(A) Quantification (right panel) of ChAT-positive L1-L2 spinal neurons (left panel) in untreated (left bars) and R-Roscovitine-treated (right bars) mice (**p<0.01, ANOVA). (B) Representative examples of excitatory synaptic inputs onto L1-L2 motoneurons in control and SMA mice. Z-stack projections of confocal images, 2 µm (5 optical sections) for the indicated genotypes and conditions (left panel). Densities of VGluT2 positive inputs per soma (right panel) (**p<0.01, ANOVA) (scale bar: 10 µm). (C) Representative images of endplates labeled with bungarotoxin (BTX) conjugated to rhodamine in control and SMA TVA muscles (left panel). Mean values of BTX surface area in all four conditions (right panel) (**p<0.01; ***p<0.001; ANOVA) (scale bar: 10 µm). (D) Ca,2.1 channel distribution in control and
Smn-deficient neuromuscular junctions (upper panel) indicated by the ratio of the P/Q-BTX area (lower panel) (*p< 0.05; ***p< 0.001, ANOVA) (scale bar: 10 µm). (E) The skeletal muscle membrane was evidenced in transversal slices (20 µm) stained against lectins with Rhodamine labeled WGA; representative images per condition (left panel) (***p< 0.01, ANOVA) (scale bar: 50 µm). Bars represent mean±SEM. The second number inside bars defines the number of mice; the first number the motoneurons in (A and B), the number of neuromuscular junctions in (C and D) and the number of myofibers in (E); n.s.: no significance. See also Figure S1A, B and C.

**Figure 3: Application of R-Roscovitine leads to enhanced spontaneous Ca$^{2+}$ transients in Smn$^{-/-};$SMN2 motoneurons.**

(A) Representative responses of control motoneurons to 40 sec. application of 5 µM S-Roscovitine followed by R-Roscovitine in the soma and the growth cone. (B) Permanent application of R- or S-Roscovitine over 5 days in culture. The left panel shows the frequency of Ca$^{2+}$ transients per neuron at the growth cone of control and SMA motoneurons untreated and under permanent R- or S-Roscovitine exposure (**p<0.01, ANOVA). The red line defines the mean values. The middle panel depicts the associated bar diagram (mean±SEM) (**p<0.01, ANOVA). The right panels show spontaneous Ca$^{2+}$ spikes indicated by red dots in their corresponding growth cones. N defines number of cells, n indicates number of experiments (N/n); n.s.: no significance. See also Figs. S2A-C and S3A.
Figure 4: Permanent exposure to R-Roscovitine supports cellular differentiation of Smn<sup>-/-</sup>;SMN2 motoneurons.

(A-C) Representative immunofluorescent images of growth cones on day 5 stained with antibodies against Ca<sub>v</sub>2.2 channels (green) and phalloidin (magenta) of control (A) and SMA motoneurons, untreated (B) or permanently treated (C) with 0.5 µM R-Roscovitine (R-Rosc.) (scale bar: 5 µm). (D) The growth cone area sizes were quantified on different concentrations (0; 0.01; 0.05; 0.1; 0.5 µM) of R-Roscovitine in SMA motoneurons (**p<0.01; ***p<0.001; ANOVA). (E) Quantification of signal intensities representing Ca<sub>v</sub>2.2 channel cluster formations at growth cones (**p<0.001; ANOVA). (F) Axon lengths of R-Roscovitine (0.5 µM) and CTX - treated (ω-CTX-MVIIC, 0.3 µM) and untreated Smn-deficient and control motoneurons cultured on laminin-221/211 or on laminin-111 on day 7 (**p<0.01; ***p<0.001; ANOVA). (G) Axon lengths of S-Roscovitine (S-Rosc.) treated Smn-deficient and control motoneurons on laminin-221/211 or laminin-111 on day 7 (*p< 0.05; ***p<0.001, ANOVA). (H) Comparison of axon lengths between GV-58 versus S- or R-Roscovitine treated SMA motoneurons on laminin-221/211 after 7 days in culture (*p< 0.05; ***p<0.001, ANOVA). Bars represent mean±SEM. Numbers inside bars define number of cells and number of experiments, respectively; n.s.: no significance. See also Figs.S3B, C, and S4A-C.

Figure 5: R-Roscovitine affects the RNA profile of Smn-deficient motoneurons.

(A) Volcano plots showing the significance of change (-log<sub>10</sub>(p-value)) and the magnitude of change (log<sub>2</sub>(fold change), log<sub>2</sub>FC) for each transcript for the indicated differential expression analyses. Significantly altered transcripts with p<0.05 are marked in red. For easier visualization data points for transcripts with log<sub>2</sub>FC < -10 or > 10 (all of which were not significantly altered) were omitted. (B) Overlap of transcripts significantly changed (p<0.05) in the indicated differential expression analyses. (C) Scatter plot showing the magnitude of...
change (log$_2$(fold change), log$_2$FC) of the transcripts indicated in (B) that are significantly altered in untreated Smn$^{+/−}$;SMN2 compared to untreated Smn$^{+/+}$;SMN2 motoneurons and also significantly altered in R-Roscovitine treated Smn$^{+/−}$;SMN2 compared to untreated Smn$^{+/−}$;SMN2 motoneurons. Note that in the scatter plot only transcripts are shown that were detectable under all conditions (351 out of the 357 transcripts indicated in (B). (D) Gene ontology (GO) term analysis of the 305 transcripts indicated by the red box in (C). See also Figure S3B, Table S1-S7.

**Figure 6: R-Roscovitine increases spontaneous and evoked neurotransmitter release at control and SMNΔ7 mouse motor nerve terminals.**

(A, B) Amplitude and frequency, respectively, of spontaneous events (mEPPs) in both genotypes with and without R-Roscovitine (100 µM) (**p<0.001, ANOVA). (C, D) Average EPP size (C) and quantum content (D) during 100 stimuli at 0.5 Hz in control and in SMA terminals with and without R-Roscovitine. (E, F) R-Roscovitine increases mean EPP amplitude and quantal content in both genotypes at low frequency of stimulation (0.5 Hz) (*p< 0.05; **p<0.01; ***p<0.001, ANOVA). (G) Representative EPP traces in control fibers before and after application of S-Roscovitine at the indicated concentrations. (H) Quantal content declines with S-Roscovitine concentrations higher than 10 µM (**p<0.001, ANOVA). Bars represent mean±SEM. N = number of muscle fibers, n = number of mice; n.s.: no significance. See also Figure S2D-F.
Figure 7: R-Roscovitine reduces facilitation and increases the number of active motor nerve terminals in control and SMNΔ7 mice.

(A) Representative EPP traces from control and mutant muscle fibers before and after the application of 100 µM R-Roscovitine. (B) Quantification of paired-pulse ratio of response amplitudes in control and mutant muscle fibers with and without R-Roscovitine (*p< 0.05, ANOVA). (C) Representation of single (left) and double (right) EPPs in a control mouse. The arrows indicate the peak of each EPP. (D) Number of active terminals in the same control fiber before and after application of R-Roscovitine. (E) Proportion of recorded fibers with double EPPs with respect to the total number of fibers recorded, in control and mutant mice (**p<0.01, ANOVA). Bars represent mean±SEM. N = muscle fibers, n = number of mice (N/n); n.s.: no significance.

Figure 8: R-Roscovitine effect on evoked neurotransmitter release requires the activation of P/Q-type calcium channels in control and SMNΔ7 mice.

(A) Representative EPP traces recorded in a control fiber in physiological solution (untreated), in the presence of ω-Agatoxin IVA (ω-Aga.), and with R-Roscovitine (R-Rosc.) (100 µM) plus ω-Agatoxin IVA (ω-Aga.) (upper panel). Ω-Agatoxin IVA reduced the mean amplitude of EPPs (left graph) and the quantal content (right graph) and later application of R-Roscovitine did not increase the amplitude of the response (**p<0.001, ANOVA). (B) Example of EPPs recorded in control and SMA mutant in control solution, with R-Rosocvitine and with ω-Agatoxin IVA plus R-Roscovitine. Terminals treated with ω-Agatoxin IVA show a complete block of the evoked response elicited in the presence of R-Roscovitine as illustrated in EPP amplitude (left graph) and quantal content (right graph), both in control (p***<0.0005, ANOVA) and in SMA mice (p***<0.0005, ANOVA).
Numbers inside bars represent the number of muscle fibers and the number of mice, respectively; n.s.: no significance.

**Table S1-S7 (related to Figure 5):** RNA-Sequencing (RNASeq) of primary Smn-deficient and control motoneurons treated with and without R-Roscovitine.

**Table S1 (related to Figure 5A):** Differential expression analysis comparing untreated Smn<sup>-/-</sup>;SMN2 and untreated Smn<sup>+/+</sup>;SMN2 motoneurons.

**Table S2 (related to Figure 5A):** Differential expression analysis comparing untreated Smn<sup>-/-</sup>;SMN2 and R-Roscovitine treated Smn<sup>-/-</sup>;SMN2 motoneurons.

**Table S3 (related to Figure 5A):** Differential expression analysis comparing untreated Smn<sup>+/+</sup>;SMN2 and R-Roscovitine treated Smn<sup>+/+</sup>;SMN2 motoneurons.

**Table S4 (related to Figure 5B):** List of transcripts significantly changed in untreated Smn<sup>-/-</sup>;SMN2 versus untreated Smn<sup>+/+</sup>;SMN2 motoneurons as well as in R-Roscovitine treated Smn<sup>-/-</sup>;SMN2 versus untreated Smn<sup>-/-</sup>;SMN2 motoneurons.

**Table S5 (related to Figure 5C):** List of transcripts significantly downregulated in untreated Smn<sup>-/-</sup>;SMN2 versus untreated Smn<sup>+/+</sup>;SMN2 motoneurons and up-regulated in R-Roscovitine treated Smn<sup>-/-</sup>;SMN2 versus untreated Smn<sup>-/-</sup>;SMN2 motoneurons.

**Table S6 (related to Figure 5D):** Gene Ontology (GO) term analysis reveals enrichment of transcripts encoding proteins with functions in neuronal differentiation, neurotransmitter transport and location at the synapse.

**Table S7 (related to Figure 5C):** List of transcripts significantly up-regulated in untreated Smn<sup>-/-</sup>;SMN2 versus untreated Smn<sup>+/+</sup>;SMN2 motoneurons and downregulated in R-Roscovitine treated Smn<sup>-/-</sup>;SMN2 versus untreated Smn<sup>-/-</sup>;SMN2 motoneurons.
A Acute application of R- or S-Roscovitine

- 40 sec. 5 μM S-Roscovitine, N=14
- 40 sec. 5 μM R-Roscovitine, N=56

B Permanent exposure of R- or S-Roscovitine over 5 days in culture

Ca^{2+} transients per min.
A. Log10(p-value) vs. log2FC for different conditions:

- $^{+/-}$ (no R-R) vs. $^{-/-}$ (no R-R)
- $^{-/-}$ (no R-R) vs. $^{-/-}$ (with R-R)
- $^{+/-}$ (no R-R) vs. $^{+/-}$ (with R-R)

Symbols: $^{+/-}$: Smn$^{+/-}$; SMN2
$^{-/-}$: Smn$^{-/-}$; SMN2

R-R = R-Roscovitine

B. Venn diagrams showing overlaps in gene expression:

- $^{+/-}$ (no R-R)
- $^{-/-}$ (no R-R)
- $^{-/-}$ (with R-R)
- $^{+/-}$ (with R-R)

Numbers:
- 2404 gene expressions
- 357 overlapping
- 612 overlapping
- 134 overlapping
- 898 overlapping

C. Scatter plot showing log2FC for $^{-/-}$ (no R-R) vs. $^{-/-}$ (with R-R) and $^{+/-}$ (no R-R) vs. $^{+/-}$ (no R-R)

$r = 0.87$

D. Heatmap of 305 transcripts up-regulated by R-Roscovitine in Smn$^{-/-}$; SMN2 motoneurons

- Molecular Function
- Cellular Component
- Biological Process

Categories:
- Synaptic part
- Anchored to membranes
- Integrated into membranes
- Plasma membrane
- Synapsin
- Neurological system process
- Neuron projection development
- Neurodevelopment
- Neuron differentiation
- Neurotransmitter transport
Highlights:

R-Roscovitine prolongs survival of SMA mice.
R-Roscovitine increases Ca\(^{2+}\) influx and growth cone size of SMA motoneurons.
R-Roscovitine beneficially affects neurotransmission in SMA motor nerve terminals.
R-Roscovitine wakes up dormant synapses of SMA motoneurons.