Defective Ca\(^{2+}\) channel clustering in axon terminals disturbs excitability in motoneurons in spinal muscular atrophy

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Proximal spinal muscular atrophy (SMA) is a motoneuron disease for which there is currently no effective treatment. In animal models of SMA, spinal motoneurons exhibit reduced axon elongation and growth cone size. These defects correlate with reduced β-actin messenger RNA and protein levels in distal axons. We show that survival motoneuron gene (Smn)-deficient motoneurons exhibit severe defects in clustering Ca\(_{\text{v2.2}}\) channels in axonal growth cones. These defects also correlate with a reduced frequency of local Ca\(^{2+}\) transients. In contrast, global spontaneous excitability measured in cell bodies and proximal axons is not reduced. Stimulation of Smn production from the transgenic SMN2 gene by cyclic adenosine monophosphate restores Ca\(_{\text{v2.2}}\) accumulation and excitability. This may lead to the development of new therapies for SMA that are not focused on enhancing motoneuron survival but instead investigate restoration of growth cone excitability and function.

Introduction

The two major forms of motoneuron disease, proximal spinal muscular atrophy (SMA) and amyotrophic lateral sclerosis, are caused by selective cell death of motoneurons. Among the mechanisms that are thought to play a central role are cell-autonomous mechanisms like oxidative stress and mitochondrial dysfunction (Pasinelli and Brown, 2006), but also nonautonomous processes such as dysregulated signaling from neighboring glial cells and contacting neurons (Boillee et al., 2006; Urushitani et al., 2006). Such mechanisms have been studied in great detail for amyotrophic lateral sclerosis (Bruijn et al., 2004). In contrast, much less is known for proximal SMA, the most common form of motoneuron disease in children and young adults (Crawford and Pardo, 1996; Swash and Desai, 2000; Talbot and Davies, 2001; Iannaccone et al., 2004). This disease is caused by homozygous loss or mutations in the telomeric copy (SMN1) of the survival of motor neuron gene (SMN; Lefebvre et al., 1995) on human chromosome 5q13. Whereas the SMN1 gene allows expression of a functionally intact full-length protein, most of the transcripts from the SMN2 gene code for a truncated protein lacking the functionally important domains at the C terminus that are encoded by exon 7 (Lorson et al., 1999; Monani et al., 1999). Nevertheless, low expression of full-length Smn protein from the SMN2 gene occurs, but this is not sufficient for compensating the defect caused by SMN1 loss, thus leading to motoneuron disease in humans. In contrast to humans, mice carry only one Smn gene, and the homozygous knockout of the Smn gene in mice is lethal in early development, even before blastocysts are formed (Schrank et al., 1997).

The Smn gene is ubiquitously expressed, thus raising the question of how reduced levels of this protein lead to specific motoneuron disease. Smn plays a role in the assembly and in recycling of spliceosomal uridine-rich small nuclear RNPs (Meister et al., 2001; Pellizzoni et al., 2002). Dysfunction of such processes should lead to severe defects in all cell types. The clinical phenotype of patients with SMA indicates that low levels of SMN protein, both in the full-length and the truncated form lacking the exon 7–encoded domains, are sufficient for development, survival, and function of most types of cells, but not for motoneurons. It has therefore been hypothesized that motoneurons are more vulnerable, possibly because they are among the biggest cells in the body and their need for proper mRNA...
expression, processing, and translation is probably higher than in other cell types (Monani, 2005). This hypothesis is supported by the observation that injection of assembled small nuclear RNP complexes into early Smn-deficient zebrafish embryos can rescue defects in motoneurons (Winkler et al., 2005).

A mouse model for SMA has been generated by introducing the human SMN2 into a mouse Smn null background (Monani et al., 2000). The phenotype of these mice closely resembles that of humans. These mice develop severe paralysis within a few days after birth and normally die by postnatal days 1 and 5. Surprisingly, the loss of motoneuron cell bodies at late stages of the disease does not exceed 20%, suggesting that most motoneurons develop normally during embryogenesis and that disease becomes apparent before the majority of motoneurons are lost. Survival of spinal motoneurons that are isolated from Smn−/−; SMN2 embryos does not differ from control motoneurons (Smn+/−; SMN2). However, they exhibit a specific defect in axon elongation that correlates with a defect in β-actin mRNA translocation to distal axons (Rossoll et al., 2003).

To study the underlying pathomechanism in Smn-deficient motoneurons, we have analyzed the functional consequences of Smn deficiency in growth cones. Smn-deficient motoneurons show defects in spontaneous excitability, and these defects correlate with reduced integration of voltage-gated Ca2+ channels (VGCCs) into axonal growth cones. Treatment with cAMP increases Smn levels in motoneurons (Fig. 1 B). It declined after day 3 both in control and Smn−/−; SMN2 motoneurons, confirming previous analyses (Gu and Spitzer, 1995), perhaps because the resting potential becomes more negative in embryonic motoneurons that are cultured for longer periods (Ziskind-Conhaim, 1988). There was no difference in local transient frequency in the cell bodies and proximal axons of control and Smn−/−; SMN2 motoneurons (Fig. 1 B).

We then investigated the frequency of such spikelike spontaneous transients in axons and growth cones (Fig. 1 C). At day 3 in culture, these transients were less frequent both in control and Smn−/−; SMN2 motoneurons, indicating that not every transient spreads from the cell body and proximal axon to distal axons at this stage. At day 4, spikelike transient frequency was similar in cell bodies and distal axons in control motoneurons. At the same stage, the frequency of spikelike spontaneous Ca2+ transients that reached the distal axons and growth cones was significantly reduced in Smn−/−; SMN2 motoneurons (0.39 ± 0.11 min−1) in comparison to control Smn+/−; SMN2 cells (0.83 ± 0.18 min−1; P < 0.05). At days 5 and 7, the frequency of these spikelike transients further declined in Smn−/−; SMN2 motoneurons (Fig. 1 C). TTX and CTX also inhibited these spontaneous Ca2+ transients on day 5 in culture (Fig. S1 A). The reduction of Ca2+ transients was >50% at 0.3 μM CTX and increased to ~80% at 1 μM CTX, indicating that influx through VGCCs is the predominant source of these fast transients of cytosolic Ca2+ (Fig. S1 A).

The time course of reduced spontaneous activity in distal axons and growth cones resembles that of axonal growth defects in these cultures. As described previously (Rossoll et al., 2003), axons in cultured Smn−/−; SMN2 motoneurons are shorter at day 5. Surprisingly, significant differences in axon elongation (P < 0.05) are not detectable at earlier stages (Fig. 1, D, E, and J). Normally, between days 3 and 4, a doubling of axon length is observed in cultures of both control and Smn-deficient motoneurons, but the difference between Smn-deficient and control cells was not significant (Fig. 1, D, F, and K; P > 0.05). Only at days 5, 6, and 7 did reduced axon length become apparent in the Smn−/−; SMN2 motoneurons (Fig. 1, D, G–I, and L–N).

These data suggest that defects in axonal Ca2+ influx precede alterations in axon growth of cultured Smn−/−; SMN2 motoneurons. However, these defects occur late, starting at E14 plus four additional days in culture (E14 + 4), at a developmental stage when maximal axon elongation has already occurred and motor endplate differentiation progresses in vivo.
Local Ca$^{2+}$ transients in axonal growth cones are enhanced by laminin-211/221 in Smn$^{-/-}$; SMN2 and control motoneurons

To follow the idea that axonal defects become apparent when Smn-deficient motoneurons get in contact with skeletal muscle, we investigated motoneurons on motor endplate–specific forms of laminin (laminin-211/221). Previous papers have shown that neurite growth of motoneurons is reduced on this substrate (Porter et al., 1995; Greka et al., 2003). Furthermore, it has been shown that the β2 chain in laminin-211 interacts with the pore-forming (Ca$_{\alpha}$) subunit of the N-type VGCC (Ca$_{\alpha}$2.2; Nishimune et al., 2004). Because embryonic neurons where they act as receptors for motor endplate–specific forms of laminin (Nishimune et al., 2004). These channels are located in axon terminals of motor endplate–specific Ca$_{\alpha}$2.2 transients was determined on day 3 from control or Smn$^{-/-}$; SMN2 embryos. *, P < 0.05; ***, P < 0.001, tested by one-way ANOVA.

They were much more frequent on laminin-211/221, both in control (0.22 ± 0.06 min$^{-1}$ on laminin-211/221 vs. 0.04 ± 0.03 min$^{-1}$ on laminin-111) and Smn$^{-/-}$; SMN2 motoneurons (0.07 ± 0.03 min$^{-1}$ on laminin-211/221 vs. 0.02 ± 0.01 min$^{-1}$ on laminin-111; Fig. 2 B). In control cultures, ~50% of these local transients in growth cones could be blocked with CTX (Fig. S1, B and C), indicating that VGCCs are also responsible for some but not all fast local transients in growth cones of isolated embryonic mouse motoneurons. The frequency of these local Ca$^{2+}$ transients was significantly lower in Smn$^{-/-}$; SMN2 motoneurons on laminin-211/221 at day 5 in culture (Fig. 2 B, P < 0.05).

Ca$_{\alpha}$2.2 is reduced in axonal growth cones of Smn$^{-/-}$; SMN2 motoneurons

In embryonic motoneurons, the N-type VGCCs are predominantly expressed (Urbano et al., 2002; Spafford and Zamponi, 2003). These channels are located in axon terminals of motor neurons where they act as receptors for motor endplate–specific forms of laminin (Nishimune et al., 2004). Because embryonic cultured Smn$^{-/-}$; SMN2 motoneurons showed reduced spontaneous Ca$^{2+}$ transients in growth cones, we investigated expression and cellular distribution of Ca$_{\alpha}$2.2 in control and Smn-deficient motoneurons (Fig. 3, A–I and M–O) using polyclonal antibodies against the α2 chain of this channel. The Ca$_{\alpha}$2.2 signal intensity was quantified as arbitrary units based on quantum levels per neuron.
intracellular Cav2.2, showed this channel to be highly concentrated in growth cones of Smn-deficient motoneurons (8.7 ± 1.1 μm/m) on laminin-211/221 in comparison with laminin-111 (n = 35/42). Despite the higher frequency on laminin-211/221, local transients on laminin-211/221 were significantly less frequent in Smn-deficient motoneurons in comparison with controls. Results represent the mean ± SEM of pooled data from three independent experiments. n, number of motoneurons that were scored in total from control or Smn+/−; SMN2 embryos. *, P < 0.05 tested by one-way ANOVA.

Figure 2. Decreased frequency of local spontaneous Ca2+ transients in growth cones of Smn+/−; SMN2 motoneurons on laminin-211/221. (A) Local Ca2+ transients in growth cones (black) were more frequent in Smn+/−; SMN2 motoneurons than in Smn−/−; SMN2 motoneurons cultured for 5 d. (B) The frequency of local spontaneous Ca2+ transients was significantly higher both for control (n = 34) and Smn-deficient motoneurons (n = 39) on laminin-211/221 in comparison with laminin-111 (n = 35/42). Despite the higher frequency on laminin-211/221, local transients on laminin-211/221 were significantly less frequent in Smn-deficient motoneurons in comparison with controls. Results represent the mean ± SEM of pooled data from three independent experiments. n, number of motoneurons that were scored in total from control or Smn+/−; SMN2 embryos. *, P < 0.05 tested by one-way ANOVA.

pixel per area in cell body, proximal axon, and growth cones. A significantly reduced signal intensity (P < 0.001) was found in growth cones of Smn-deficient motoneurons (8.7 ± 1.1 μm/m) versus controls (21.9 ± 2.1 μm) (Fig. 3). A-C). Applying a fixation protocol without detergent and shortened exposure to 4% PFA to increase the staining intensity of cell surface–exposed versus intracellular Ca2.2, showed this channel to be highly concentrated in protrusions of control growth cones (Fig. 3, F and G) but not in Smn−/−; SMN2 growth cones (Fig. 3, H and I). Furthermore, Ca2.2 channels colocalize with the active zone protein Piccolo, indicating that clusters of Ca2.2 have formed active zone-like structures in the growth cone protrusions in control motoneurons (Fig. S2, A, B, C, E, and G, available at http://www.jcb.org/cgi/content/full/200703187/DC1). The colocalization of Ca2.2 with Piccolo was highly reduced in Smn-deficient growth cones (Fig. S2, B, D, F, and H). In control motoneurons, these active zone-like structures cover ~20% of the whole growth cone area. In Smn-deficient motoneurons <5% of the active zone-like structures are detectable (Fig. S2 I). These structures are only found in protrusions but not the core or the proximal parts of growth cones and axons (Fig. S2, J-L). In contrast to growth cones, the signal intensity in cell bodies of Smn+/−; SMN2 and Smn−/−; SMN2 motoneurons was not different (Fig. 3, A, B, and E).

To analyze whether the reduced Ca2.2 expression in distal axons is caused by disturbed subcellular distribution of the corresponding mRNA, in situ hybridization was performed. This experiment did not reveal any difference in cellular distribution or rough differences in expression levels between control and Smn-deficient motoneurons (Fig. 3, J and K). The specificity of the in situ hybridization was controlled with a Ca2.2 sense probe (Fig. 3 L).

We also applied stimulated emission depletion (STED) fluorescence microscopy (Dyba and Hell, 2003; Kittel et al., 2006; Willig et al., 2006) to investigate whether the reduced accumulation of Ca2.2 reflects a defect in cluster formation of this channel. This method enhances the resolution of confocal microscopy in the xy axis, so that structures <200 nm that normally cannot be resolved by classical light microscopy become detectable. Intracellular vesicles containing Ca2.2 channels are much smaller than the Ca2.2 clusters that form on the cell surface. When we compared the size of the Ca2.2 immunoreactive areas in control and Smn+/−; SMN2 growth cones, it became apparent that in Smn-deficient growth cones the relative density of large clusters covering an area of at least 0.01 μm2 versus small vesicles is reduced compared with control growth cones (Fig. 3, M–O). Collectively, these data suggest that a defect in Ca2.2 transfer into the cell membrane and active zone-like structures in Smn-deficient growth cones is responsible for reduced frequency of Ca2+ transients.

Inhibition of axon elongation by laminin-211/221 does not occur in Smn+/−; SMN2 motoneurons

In parallel to Ca2+ transients, we also analyzed axon elongation on laminin-211/221. Mean axon length of control motoneurons was 264.1 ± 11.2 μm on laminin-211/221 in comparison to 335.2 ± 19.0 μm on laminin-111 after 7 d in culture (Fig. 4, A, B, and D). Surprisingly, Smn-deficient motoneurons did not show such a reduction of axon growth on laminin-211/221. In contrast, they exhibited a slight but significant (P < 0.05) increase in axon extension (309.2 ± 12.5 μm) in comparison with control motoneurons (259.6 ± 10.3 μm) on laminin-211/221 (Fig. 4, A, C, and E).

We then measured the size of axonal growth cones because previous studies have shown that the deficit in axonal β-actin mRNA and protein correlates with reduced axon elongation and growth cone size in Smn−/−; SMN2 motoneurons that were cultured on laminin-111 (Rossoll et al., 2003). The growth cone area of Smn-deficient motoneurons on laminin-211/221 did not differ from the area on laminin-111 (Fig. 4, F, H, and J). On both substrates, growth cones of Smn−/−; SMN2 motoneurons were smaller than those of Smn+/−; SMN2 motoneurons (Fig. 4, F–J).

Blockade of N-type Ca2+ channels does not reduce axon growth in Smn-deficient motoneurons

Inhibition of Ca2.2 with CTX blocks global Ca2+ transients in control motoneurons by >80% (Fig. S1 A). In addition, local transients that only occur in axonal growth cones are reduced by >50%, both at 1 and 0.3 μM CTX, which is considered to be highly specific for N-type VGCCs (Fig. S1, A–C). Interestingly, the reduction of local transients in growth cones is smaller, thus confirming earlier observations with Xenopus laevis motoneurons that other Ca2+ channels contribute to rapid local Ca2+
transients in growth cones (Spitzer et al., 2000). To determine the role of classical VGCCs for axon growth, we tested whether specific blockade of Ca_{2.2} with CTX affects axon growth of motoneurons in vitro. CTX was applied at 1 μM and a lower concentration (0.3 μM) that is considered highly specific for N-type VGCCs (Figs. 5 and S1). After 7 d in culture, motoneurons grown on laminin-111 or laminin-211/221 were fixed and stained against tau and microtubule-associated protein 2 to distinguish dendrites and axons (Rossoll et al., 2003). 1 μM CTX led to an increase in axon growth in control motoneurons at both concentrations, whereas 0.3 μM CTX treatments (Fig. 5 B), that probably (as discussed in the text) reflect clusters at the cell surface (arrows). The ratio of vesicles and clusters is increased in Smn-deficient motoneurons, caused by a significant reduction of the cluster-like structures in Smn+/+; SMN2 embryos (n = 35) in comparison to controls (n = 32). (L) The specificity of the antisense probe was tested with a sense Ca_{2.2} cDNA probe. (M and N) For high-resolution microscopy, STED microscopy was applied to reduce the focal spot area by one order of magnitude, thus allowing the distinction of small vesicle-like structures (arrowheads) from larger areas that probably (as discussed in the text) reflect clusters at the cell surface (arrows). (O) The ratio between vesicles and clusters in Smn+/+; SMN2 growth cones (N and O) in contrast to growth cones of control motoneurons (M and O). Results represent the mean ± SEM of pooled data from three independent experiments. n, number of motoneurons that were scored in total from control or Smn+/−; SMN2 embryos. *, P < 0.05; **, P < 0.001, tested by one-way ANOVA and t-test, respectively.

Figure 3. Expression and cellular distribution of Ca_{2.2} protein and mRNA in primary motoneurons of Smn+/−; SMN2 and Smn−/−; SMN2 embryos. [A–E] Distribution and quantitative analysis of Ca_{2.2} protein in 7-d-old primary motoneurons of Smn+/−; SMN2 and Smn−/−; SMN2 embryos on laminin-211/221. The signal intensity for Ca_{2.2} protein in the cell body of Smn+/−; SMN2 motoneurons (A and E; n = 30) is not reduced in comparison to control cells (B and E; n = 30). In contrast, the signal intensity for Ca_{2.2} is lower in axonal growth cones of Smn+/−; SMN2 motoneurons (D and E; n = 30) in comparison to controls (C and E; n = 30). (J–K) Differences in Ca_{2.2} mRNA distribution are not detected in cell bodies (arrows) and growth cones (arrowheads and insets) of motoneurons from Smn+/−; SMN2 embryos (n = 35) in comparison to controls (n = 32). (L) The ratio between vesicles and clusters in Smn+/+; SMN2 motoneurons (A and E; n = 30) is not reduced in comparison to controls (B and E; n = 30). STED microscopy was applied to reduce the focal spot area by one order of magnitude, thus allowing the distinction of small vesicle-like structures (arrowheads) from larger areas that probably (as discussed in the text) reflect clusters at the cell surface (arrows). The ratio of vesicles and clusters in Smn+/+; SMN2 growth cones (N and O) in contrast to growth cones of control motoneurons (M and O). Results represent the mean ± SEM of pooled data from three independent experiments. n, number of motoneurons that were scored in total from control or Smn+/−; SMN2 embryos. *, P < 0.05; **, P < 0.001, tested by one-way ANOVA and t-test, respectively.
In contrast, axons of Smn−/−; SMN2 motoneurons on laminin-211/221 (Fig. 6 A). In contrast, axons of Smn−/−; SMN2 motoneurons (n = 210) are shorter than those of control motoneurons (n = 93) on laminin-111. (A, D, and E) In contrast, axons of Smn−/−; SMN2 motoneurons (n = 141) extend significantly longer on laminin-211/221. (F–J) Growth cones of Smn-deficient motoneurons are significantly smaller both on laminin-111 (F–H; n = 32) and laminin-211/221 (F, I, and J; n = 30). Results represent the mean ± SEM of pooled data from three independent experiments. n, number of motoneurons that were scored in total from control or Smn−/−; SMN2 embryos. *, P < 0.05; **, P < 0.001, tested by one-way ANOVA.

8-(4-chlorophenylthio)-3′,5′ cAMP (8-CPT-cAMP) enhances SMN protein in Smn−/−; SMN2 motoneurons and restores morphological and functional alterations in axons

We further tested whether cAMP, which has previously been described to enhance the frequency of spontaneous Ca2+ transients in developing Xenopus motoneurons (Gorbunova and Spitzer, 2002), could enhance the frequency of Ca2+ transients in growth cones of control and Smn-deficient mouse motoneurons. Surprisingly, there was only little effect (P > 0.05) in cultured control motoneurons (Fig. 6 A). In contrast, Smn−/−; SMN2 axonal growth cones react with a more than twofold increase in the frequency of Ca2+ transients on day 5 in axon terminals after treatment with 100 μM 8-CPT-cAMP. To test whether 8-CPT-cAMP has a direct effect on Ca2+ transients, we added CTX together with 8-CPT-cAMP (Fig. S1, C and D). Enhanced cAMP does not rescue the reduction of Ca2+ transients that is caused by CTX (Fig. S1 C), indicating that there are no CTX-insensitive channels present that open in response to 8-CPT-cAMP. When we treated Smn−/−; SMN2 motoneurons with CTX, there was little further reduction of local Ca2+ transients, but treatment with 8-CPT-cAMP increases Ca2+ transient frequency (Fig. S1 D). This improvement only occurs in Smn−/−; SMN2 but not in control motoneurons, leading to similar levels of Ca2+ transients than those observed with CTX and 8-CPT-cAMP in control motoneurons (Fig. S1 C). The difference between control and Smn-deficient motoneurons suggests that the improvement is not caused by the opening of VGCCs that were blocked by CTX treatment, because this increase in Ca2+ transients should then occur in both groups and not only in Smn−/−; SMN2 motoneurons.

We then analyzed whether this increase of Ca2+ transients in 8-CPT-cAMP–treated Smn-deficient motoneurons correlates with increased Ca2,2.2 expression in Smn-deficient growth cones. Both the signal intensity of the Ca2,2.2 staining (Fig. 6, B–D, F, G, I, and J) and β-actin in growth cones (Fig. 6, B, C, E, F, H, I, and K) was increased to almost normalized levels by this treatment.

The Smn promoter region contains a CreII-binding element (Majumder et al., 2004) that mediates cAMP effects on increased SMN2 transcription in mouse hepatocytes. We therefore
tested whether cAMP up-regulates Smn expression and thus restores the morphological and functional alterations in Smn-deficient motoneurons. For this purpose we analyzed Smn protein levels and distribution in cultured embryonic motoneurons. Because the number of motoneurons that can be isolated from one Smn-deficient embryo is not sufficient for quantitative RT-PCR or Western blot analysis, we prepared protein and RNA extracts from cultures of E11.5 forebrain neuronal precursor cells from control and Smn-deficient mice. 100 μM 8-CPT-cAMP increased SMN2 mRNA (Fig. S4, A and B, available at http://www.jcb.org/cgi/content/full/jcb.200703187/DC1) and protein levels by ~40–100% (Fig. S4 C) in these cells.

Primary motoneurons from Smn−/−; SMN2 and Smn−/+; SMN2 embryos were treated with 100 μM 8-CPT-cAMP. Both in cell bodies, axons, and axonal growth cones, Smn-specific fluorescence signal intensity was enhanced in Smn-deficient neurons but not fully restored to control levels (Fig. 7, A–J). The reduction of growth cone size is the most prominent pathological feature in cultured Smn−/−; SMN2 motoneurons (Rossoll et al., 2003). 8-CPT-cAMP treatment normalized growth cone size in Smn−/−; SMN2 motoneurons to control levels and CTX did not abolish the rescue effect, indicating that it does not involve enhanced Ca2++ transients (Fig. 8, A–E). We then investigated whether this effect is caused by a normalization of local β-actin levels in distal axons. The ratio of distal to proximal β-actin protein levels is reduced in Smn−/−; SMN2 motoneurons (Rossoll et al., 2003), and this ratio was normalized by 8-CPT-cAMP (Fig. 8 F). The altered β-actin ratio is based on increased actin mRNA in the growth cone of 8-CPT-cAMP–stimulated Smn-deficient motoneurons (Fig. 8, G–N). Thus, elevated cAMP increases...
distal actin mRNA and protein levels in axons, leading to augmented Ca,2,2 levels in growth cones and normalization of Ca²⁺ transient frequency. Subsequently, we tested whether this effect also rescues responsiveness to laminin-211/221 in the Smn⁻/⁻; SMN2 motoneurons. As shown in Fig. 8 (O–R), axon length is shortened in Smn⁻/⁻; SMN2 neurons on laminin-211/221 when the cells are treated with 8-CPT-cAMP, indicating that the responsiveness to motor endplate-specific forms of laminin is restored by elevated cAMP levels.

**Discussion**

We have investigated the correlation between defective axon elongation and spontaneous excitability in motoneurons from a mouse model of SMA. We observed that the reduction of spontaneous Ca²⁺ transients in distal axons and growth cones is caused by defective Ca,2,2 accumulation and clustering in the axonal growth cones, thus influencing axon growth in the Smn-deficient motoneurons. These defects can be at least partially compensated by 8-CPT-cAMP treatment. Motoneuron disease in SMA, both in humans and mouse models, becomes apparent after motoneurons have made contact with skeletal muscle. In a mouse model of SMA type I, motoneuron loss is not enhanced during a critical period of development when motoneurons depend on trophic support from target tissues (Monani et al., 2000). Motoneuron numbers are normal at birth, but decrease at postnatal days 3–5. During this period, differentiation of motor endplates takes place, and we therefore investigated neurons that were cultured on mo-

Gene knockout mice for the laminin β2 chain (Lamb2⁻/⁻) or Ca,2,1 (Ca,2,1⁻/⁻) exhibit strong synaptic maturation defects. Lamb2-deficient mice develop neuromuscular junction (NMJ) degeneration, which is characterized by disturbed active zones just after birth (Ino et al., 2001; Nishimune et al., 2004). Ca,2,2 knockout mice do not show any signs of motoneuron disease, as the defect can most probably be compensated by Ca,2,1 expression. Ca,2,1-deficient mice develop normally until the third postnatal week (Jun et al., 1999). But from this time point on, as in Lamb2⁻/⁻ mice, the NMJs degenerate and exhibit a decrease of active zone proteins (Nishimune et al., 2004; Fox et al., 2007). The delayed disease onset in Ca,2,1-deficient mice can be explained by a compensatory effect of residual Ca,2,2, which is substituted postnataally by Ca,2,1. Altogether, these data indicate that the β2 chain interaction of laminin-221 with Ca,2,1 and Ca,2,2 supports postnatal development and maintenance of NMJs (Nishimune et al., 2004). Our data suggest that the reduced Ca,2,2 accumulation in the axonal growth cone protrusions of SMN2; SMN2 motor axons (n = 82) show reduced distal β-actin signal intensity in comparison to control cells (n = 78). 8-CPT-cAMP treatment of Smn⁻/⁻; SMN2 motoneurons restores distal β-actin levels [checkered gray bar; (G–N) Analysis of actin mRNA levels in axonal growth cones by in situ hybridization in motoneurons from Smn⁻/⁻; SMN2 (G, I, and L; n = 30), 8-CPT-cAMP-treated Smn⁻/⁻; SMN2 (G, J, and M; n = 32), and control embryos (G, H, and K; n = 30)]. 8-CPT-cAMP restores the actin mRNA deficit in axon terminals of Smn⁻/⁻; SMN2 motoneurons. (N) A sense actin probe was used as a negative control for the specificity of the actin probe. (O–R) 8-CPT-cAMP also restores the response of Smn⁻/⁻; SMN2 motor axons to laminin-211/221. Axon elongation after 7 d on laminin-211/221 in control (P; n = 141), Smn⁻/⁻; SMN2 (Q; n = 141) and 8-CPT-cAMP-treated Smn⁻/⁻; SMN2 (R; n = 60) motoneurons. (O) Quantitative analysis of data. Results represent the mean ± SEM of pooled data from three independent experiments. n, number of motoneurons that were scored in total from control, Smn⁻/⁻; SMN2 or Smn⁻/⁻; SMN2 embryos were treated with 100 μM 8-CPT-cAMP. * P < 0.05; ** P < 0.001, tested by one-way ANOVA.

![Figure 8. 8-CPT-cAMP restores axon elongation, growth cone size, and responses to laminin-211/221 in Smn⁻/⁻; SMN2 motoneurons.](image-url)
Smn-deficient motoneurons is responsible for reduced axon elongation in cell culture and reduced responsiveness to synapse-specific laminin isoforms.

**Mislocalization of N-type Ca\(^{2+}\) channels in Smn-deficient motoneurons on laminin-211/221 leads to local excitability defects at the growth cone**

In patients with SMA, extended neuromuscular latency to electrical stimulation is observed (Krajewska and Hausmanowa-Petruzel, 2002), pointing to defects in synaptic transmission at motor endplates. SMA is a dying-back disease in which the degeneration of motoneurons starts at the presynaptic part and proceeds backward to the cell body (Cifuentes-Diaz et al., 2002). Fly mutants lacking Smn show reduced excitatory postsynaptic currents and disorganized synaptic boutons, pointing to defects in synaptic function at NMJs (Chan et al., 2003). Such fly models provide further evidence for disturbed actin metabolism (Rajendra et al., 2007).

Target-derived signals play a critical role for presynaptic differentiation and maintenance during the period when synapses are eliminated and polysynaptic innervation of skeletal muscle fibers is removed (Nguyen and Lichtman, 1996). β2-containing laminin isoforms act as signals for presynaptic differentiation (Son et al., 1999). The disturbed presynaptic synthesis of β-actin in Smn-deficient motoneurons interferes with VGCC translocalization to membrane clusters, which, in turn, could impair transmitter release from the axon terminals (Zhong and Zucker, 2004). Interestingly, other types of Ca\(^{2+}\) channels such as TRPC5 and 6 are not affected. These channels do not cluster on growth cone protrusions and are distributed more widespread over the growth cone. Moreover, double staining with Piccolo, a component of the presynaptic apparatus, revealed a severe defect of Ca,2.2 accumulation into active zone-like structures of Smn-deficient motoneurons.

Control motoneurons show reduced axon elongation on laminin-211/221 that correlates with increased Ca\(^{2+}\) spike frequency in axonal growth cones. The contribution of Ca,2.2 accumulation and subsequent enhanced frequency of Ca\(^{2+}\) transients to axon elongation on laminin-211/221 becomes evident by CTX treatment. Ca,2.2 inhibition restores axon elongation on laminin-221/211. The lack of response of Smn-deficient motoneurons to laminin-211/221 could be explained by a massive reduction of Ca\(^{2+}\) transients in the growth cone.

Collectively, we conclude that Smn-deficient motoneurons are not able to differentiate in response to synapse-specific laminins because of their incapability of accumulating Ca,2.2 in axon terminals. Thus, defective Ca\(^{2+}\) channel accumulation could lead to dysfunction of the active zone (Nishimune et al., 2004), and this in turn could lead to disturbed neurotransmitter release and thus to degeneration of NMJs.

**cAMP compensates for the morphological and functional defects in Smn-deficient motoneurons**

We also found that 8-CPT-cAMP restored the defect in excitability in axon terminals of Smn\(^{-/-}\); SMN2 motoneurons. 8-CPT-cAMP treatment enhances spontaneous Ca\(^{2+}\) influx into spinal motoneurons of Xenopus oocytes through VGCCs (Gorbunova and Spitzer, 2002). In addition, the ratio of cAMP to cGMP has been reported to regulate polarity in netrin-1–induced axon guidance in Xenopus spinal motoneurons (Nishiyama et al., 2003). The enhanced frequency of spontaneous Ca\(^{2+}\) transients in axonal growth cones of Smn\(^{-/-}\); SMN2 motoneurons correlates with increased Ca,2.2 accumulation in growth cones. It appears as if this defect is reversed by increased β-actin protein level in distal axons of Smn\(^{-/-}\); SMN2 motoneurons. Thus, our results with 8-CPT-cAMP could guide the way to new therapeutic strategies for SMA.

**Materials and methods**

**Motoneuron culture**

The ventrolateral part of the lumbar spinal cord of E14 embryos was dissected and transferred to HBSS. After 15 min of treatment with 0.05% trypsin, cells were triturated and cultured after enrichment by panning with antibodies against the mouse p75 neurotrophin receptor (Abcam). Cells were plated at a density of 2,000 cells/cm\(^2\) in 4-well dishes (Greiner Bio-One) and cultured as described previously (Wiese et al., 1999). The culture dishes were precoated with polyornithine and laminin-111 or laminin-211/221 (Invitrogen), respectively. 100 μM 8-CPT-cAMP (dissolved in HBSS, Calbiochem), 1 μM TTX (Sigma-Aldrich), and 1 or 0.3 μM CTX (Sigma-Aldrich), respectively, was added by changing the medium every second day.

**Forebrain neuronal precursor cell culture**

Precursor cells from the forebrain of 11.5-d-old mouse embryos were prepared, and neurospheres were cultured in neurobasal medium with 500 μM glutamax (Invitrogen), 50 μM penicillin G sodium and 50 μM streptomycin sulfate (Invitrogen), 2% FBS (1:50; Invitrogen), and 20 ng/ml EGF and bFGF (Cell Concepts).

For Western blot analysis, the neurospheres were plated at high density on 60-mm dishes that had been coated with polyornithine and laminin-111. These cells were then grown for 72 h.

**Antibodies used for immunocytochemistry**

Immunocytochemistry was performed as described previously (Rassoul et al., 2003). For analysis of membrane-exposed N-type Ca\(^{2+}\) channels, we fixed the cells only for 2 min with 4% PFA in 1× TBS without acetone. In addition, Tween 20 (Sigma-Aldrich) was omitted from all buffers for this set of experiments (Fig. 3, F–I). The following primary antibodies were used: rabbit polyclonal antibodies against tau at 1 μg/ml (1:1,000; Sigma-Aldrich); an N-type Ca\(^{2+}\) channel (1:200; Sigma-Aldrich); TRPC5 (1:200, Sigma-Aldrich), and TRPC6 (1:200, Chemicon), mouse monoclonal antibodies against 1 μg/ml β-actin (Abcam), 1 μg/ml microtubule-associated protein 2 (Sigma-Aldrich), and 2 μg/ml Smn (BD Biosciences). Cells were then washed three times with 1× TBS-T (20 mM Tris-Cl, pH 7.6, 137 mM NaCl, and 0.1% Tween 20) and incubated for 1 h at room temperature with Cy2 (1:200)- and Cy3 (1:300)-conjugated secondary antibodies (DianoV). The Alto 647N–conjugated secondary antibody (Alto Technology) was used for STED microscopy. Confocal images were obtained either with a microscope (TCS 4D; Leica) with a 20× 0.5 objective (PL FLUOTAR) or a microscope (SP2; Leica) with a 100× 1.4 oil-immersion objective (HCX PL APO CS), with identical settings for pinhole and voltage for control and treated samples. For high-resolution microscopy, a STED setup mounted to a microscope (SP5; Leica) with a 100× 1.4 oil immersion objective was applied.

**Western blot analysis**

Forebrain neuronal precursor cells grown on laminin-111 for 72 h were collected from the dishes, and protein extraction for Western Blotting was performed as described previously (Rassoul et al., 2002). Primary antibodies, 1 μg/ml anti–mouse β-actin antibody (Abcam), 1 μg/ml anti–mouse β-III tubulin antibody (RDI), and 2 μg/ml Smn IgG1 (BD Biosciences) were used.

**Calcium imaging**

For Ca\(^{2+}\) imaging analysis, cultured motoneurons were grown on glass coverslips. After gently washing in phenol red-free HBSS and permeabilization with 0.25% pluronic F-127 (Sigma-Aldrich) over 5 min, cells were
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Online supplemental material

Fig. S1 shows that Ca2+ transient frequency is reduced by TTX and CTX in cultured motoneurons from 14-d-old mouse embryos. Fig. S2 shows co-localization of Ca.2.2 and the presynaptic protein Piccolo in protrusions of axonal growth cones in control and Smn−/−, SMN2 motoneurons. Fig. S3 shows distribution and semiquantitative analysis of TRPC5 and δ immuno-
reactivity in the growth cones of control and Smn-deficient motoneurons. Fig. S4 shows that B-CFTR-CAMP stimulates SMN2 and Smn transcription and increases Smn protein levels in Smn-deficient forebrain neuronal pre-
cursor cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/200703187/DC1.

Total RNA from neuronal precursor cells was extracted by Trizol (Invitro-
gen) according to the manufacturer’s protocol, and 1 μg of total RNA was used for cDNA amplification. Amplification of the SMN2 cDNA was per-
formed with Ex 5f (5′-CCCTACTATGATGGCT-3′) and Ex 8r (5′-CATACAC-
ACCCCTCTTCAAG-3′) primers under the following PCR conditions: 3 min at 94°C (1 cycle), 30 s at 94°C, 30 s at 56°C, 45 s at 72°C (30 cycles), and 5 min at 72°C (1 cycle).

For the quantification of β-actin and N-type Ca2+ channel distribution within the different cellular compartments, the staining intensity in the cell body, the proximal and the distal third of the axon, and the growth cone were analyzed with AIDA software (Raytest). Background intensity was measured for every single picture. The intensity for β-actin and N-type Ca2+ channels was measured as arbitrary units per area, based on quan-
tum levels per pixel, according to the manufacturer’s instructions.

For STED microscopy, LAS AF acquisition software (modified by Leica) was used. The deconvolution processing was performed with Inspector (Max-Planck Institute).

The final processing of all images was performed with Photoshop 7.0 (Adobe) and Illustrator 10 (Adobe). Linear contrast enhancement was applied to Figs. 1 (E–N), 4 (B–E), and 8 (P–R), and all individual panels in these figures were treated similarly.

Values from at least three independent experiments were pooled and the results were expressed as the mean ± SEM. Statistical significance of differences were assessed by one-way analysis of variance (ANOVA) and Bonferroni after testing using Prism software (GraphPad).
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