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$_{v}$2.2 channels in axonal growth cones. These defects also correlate with a reduced frequency of local Ca$^{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$_{v}$2.2 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; Urunishi 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 day 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 Smn−/−; SMN2 motoneurons and leads to restoration of the morphological and functional deficits in axons and axon terminals. These findings indicate that reduced excitability in growth cones contributes to the disease phenotype. This defect could lead to disturbances of active zones in the presynapse, causing reduced transmitter release at the motor endplate that, in turn, could contribute to motoneuron malfunction and degeneration in SMA.

Results

Spontaneous excitability is reduced in cultured Smn-deficient motoneurons

In isolated embryonic Smn−/−; SMN2 motoneurons, translocation of β-actin mRNA to distal axons and growth cones is disturbed (Rossoll et al., 2003). To investigate the functional consequences of Smn deficiency in axon terminals, we measured spontaneous excitability in motoneurons that were isolated from embryonic day (E) 14 of control and Smn−/−; SMN2 mutant mouse embryos, and cultured them on laminin-111. The cultured neurons were loaded with Fura-2, a Ca2+-binding fluorescent dye (Fig. 1 A, top left) and analyzed over periods of 7.5 min at 3, 4, 5, and 7 d in culture (Fig. 1 A, bottom left). In parallel to these measurements, motoneuron survival in the presence of brain-derived and ciliary neurotrophic factor (10 ng/ml each) was determined. No difference was observed between Smn-deficient and control motoneurons (Rossoll et al., 2003). In control Smn+/+; SMN2 motoneurons that were cultured on laminin-111, spontaneous spikelike Ca2+ transients were detectable that appeared synchronized in the cell body, dendrites, axons, and axonal growth cones, thus reflecting global spontaneous depolarization as described previously for developing neurons (Gu et al., 1994; Ciccolini et al., 2003). These transients could be blocked by 1 μM tetrodotoxin (TTX), indicating that they are triggered by opening of voltage-gated Na+ channels (Fig. 1 A, top right). The addition of 0.3 and 1.0 μM ω-conotoxin MVIIA (CTX) inhibited these Ca2+ transients, suggesting that N-type Ca2+ channels are responsible for the Ca2+ transients measured in these cultures (Figs. 1 A, bottom right; and S1 A), available at http://www.jcb.org/cgi/content/full/200703187/DC1).

The frequency of spikelike global spontaneous Ca2+ transients within cell bodies and proximal axons of control motoneurons (Smn+/+; SMN2) was highest on day 3, both in Smn−/−; SMN2 and control 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-221 interacts with the pore-forming (Ca$_s$) subunit of the N-type VGCC (Ca$^{2+}$; Nishimune et al., 2004). Thus, presynaptic differentiation appears mediated through the direct interaction of laminin-221 with Ca$_s$. We therefore measured Ca$^{2+}$ through the direct interaction of laminin-221 with Cav2.2 channels.

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 motoneurons 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$_{s}$ 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$_s$ signal intensity was quantified as arbitrary units based on quantum levels per body, were barely detectable on laminin-111, neither in Smn$^{-/-}$; SMN2 nor in control motoneurons at day 5 in culture (Fig. 2 B).

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$_{2.2}$ 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 motoneurons 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$_{2.2}$ 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$_{2.2}$ signal intensity was quantified as arbitrary units based on quantum levels per
increase the staining intensity of cell surface–exposed versus
sus controls (21.9 ± 2.1; Fig. 3, C–E). Applying a fixation pro-
posal, Cav2.2 channels colocalize with the active zone
localization of Cav2.2 with Piccolo was highly reduced in Smn-
(http://www.jcb.org/cgi/content/full/200703187/DC1). The co-
active zone-like structures in the growth cone protrusions in
sm-deficient motoneurons (8.7 ± 1.1) ver-
in growth cones of Smn-deficient motoneurons (8.7 ± 1.1) ver-
(II, F11601). Despite similar growth cone area of Smn-deficient
cone area of Smn-deficient motoneurons on laminin-211/221
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
ferent frequency on laminin-211/221, local transients on laminin-
neurons were cultured for 5 d. (B) The frequency of local spontaneous Ca2+
transients was significantly higher for control (n = 34) and Smn-deficient motoneurons (n = 39) on laminin-211/221 in comparison with laminin-111 (n = 35/42). Despite
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ferent frequency on laminin-211/221, local transients on laminin-
and Smn-deficient motoneurons (Fig. 3, J and K). The specificity
of the in situ hybridization was controlled with a Ca,2,2 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 Ca,2,2 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 nor-
mally cannot be resolved by classical light microscopy become
detectable. Intracellular vesicles containing Ca,2,2 channels are
much smaller than the Ca,2,2 clusters that form on the cell
surface. When we compared the size of the Ca,2,2 immunoreactive
areas in control and Smn−/−; SMN2 growth cones, it became ap-
parent 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 Ca,2,2
transfer into the cell membrane and active zone-like structures
in Smn-deficient growth cones is responsible for reduced fre-
quency of Ca2+ transients.

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. In
contrast to growth cones, the signal intensity in cell bodies of
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
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ferent frequency on laminin-211/221, local transients on laminin-
ment and growth cone size in
β-actin mRNA and protein correlates with reduced axon elon-
gation 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
 skłon by 80% (Fig. S1 A). In addition, local
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 moto-
neurons 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 Ca,2,2 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 moto-
neurons 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\textsubscript{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 specifically reduces axon growth of control motoneurons on laminin-111 (335.2 ± 19.0 vs. 240.7 ± 8.5 μm), whereas 0.3 μM CTX was less efficient (335.2 ± 19.0 vs. 288.2 ± 11.8 μm; Fig. 5 A). Axon growth of Smn-deficient neurons on laminin-111 (233.4 ± 13.8 μm), which was already disturbed, was not further reduced both by 1 and 0.3 μM CTX (Fig. 5 A).

We then investigated the effect of 0.3 and 1 μM CTX on motoneurons grown on laminin-211/221. Inhibition of Ca\textsubscript{2.2} with CTX led to an increase in axon growth in control motoneurons (Fig. 5 B) at both concentrations. Axon elongation reached comparable levels to those seen in motoneurons on laminin-111 (Fig. 5 A). Smn-deficient motoneurons did not show increased axon elongation after 0.3 and 1 μM CTX treatments (Fig. 5 B), indicating that the response of axonal growth to blockade of N-type Ca\textsuperscript{2+} channels is lacking in Smn-deficient motoneurons. Low concentrations (0.3 μM) of CTX showed similar effects as 1 μM CTX on laminin-211/221, thus providing an argument for the specificity of the effect for Ca\textsubscript{2.2} channels.

In other types of neurons, TRPC3 and 6 have been shown to mediate Ca\textsuperscript{2+} fluxes that modulate axon guidance and neuronal survival in response to brain-derived neurotrophic factor (Li et al., 2005; Jia et al., 2007). In addition, TRPC5 activation promotes axon guidance and neurite growth in hippocampal neurons (Grek et al., 2003). Therefore, the possibility exists that TRPCs are also involved in the pathological alterations observed in Smn-deficient motoneurons. Among these candidates, TRPC5 is highly expressed in embryonic motoneurons. TRPC6 is also found but at relatively lower levels, whereas TRPC3 is barely detectable (unpublished data). When we analyzed the distribution of TRPC5 and 6 immunoreactivity in growth cones of control and Smn-deficient motoneurons, no difference was observed, both with respect to subcellular distribution (Fig. S3, A–D, available at http://www.jcb.org/cgi/content/full/200703187/DC1) and signal intensity (Fig. S3, E and F). This led us to conclude that alteration in Ca\textsubscript{2.2} distribution is responsible for the differences in Ca\textsuperscript{2+} transients observed in growth cones of Smn-deficient motoneurons.
In contrast, axons of Smn−/−; SMN2 motoneurons on laminin-211/221 were significantly shorter than those of control motoneurons (P < 0.001, tested by one-way ANOVA). 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 (I, 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 < 0.001, tested by one-way ANOVA.

Figure 4. Axon growth and growth cone morphology of Smn−/−; SMN2 motoneurons on laminin-211/221. (A–E) Axon length of control and Smn-deficient motoneurons on laminin-111 and laminin-211/221 after 7 d in culture. (A–C) 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 (I, 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 < 0.001, tested by one-way ANOVA.

Figure 5. Axon growth after blockade of Ca2+2.2, with CTX in Smn−/−; SMN2 and control motoneurons. (A and B) Axon length of 7-d-old isolated motoneurons from control and Smn-deficient embryos that were grown on laminin-111 (A) and laminin-211/221 (B) treated with 1 and 0.3 μM CTX, respectively. (A) On laminin-111, control neurons responded to 1 (n = 162) and 0.3 μM CTX (n = 186) with reduced axon growth, whereas Smn-deficient cells were unaffected in the presence of both concentrations (n = 151 for 1 μM and n = 82 for 0.3 μM CTX). (B) On laminin-211/221, CTX-treatment, both with 1 and 0.3 μM, increases axon extension in control motoneurons (n = 99 for 1 μM and n = 59 for 0.3 μM CTX) but not in Smn-deficient neurons (n = 102 for 1 μM and n = 82 for 0.3 μM CTX). 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 < 0.001, tested by one-way ANOVA.
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 but not fully restored to control levels (Fig. 8, A–I). 8-CPT-cAMP treatment normalized both β-actin levels and distribution in growth cones of Smn−/−; SMN2 motoneurons (Fig. 8, G–N). Thus, elevated cAMP increases

![Figure 6](image1.png)

**Figure 6.** 8-CPT-cAMP treatment restores local Ca2+ transients and N-type Ca2+ channel (Ca2.2) immunoreactivity in axonal growth cones of Smn−/−; SMN2 motoneurons. (A) Local Ca2+ transients in growth cones of Smn-deficient motoneurons (n = 30) that were cultured for 5 d on laminin-211/221 are restored by 8-CPT-cAMP in comparison with controls (n = 30). (B) Quantitative analysis of immunoreactivity for Ca2.2 and β-actin in 5-d-old control (white bar; n = 30), Smn−/−; SMN2 (gray bar; n = 30), and 8-CPT-cAMP-treated Smn−/−; SMN2 motoneurons (checkered bar; n = 30) cultured on laminin-211/221. [C–E] In control motoneurons, Ca2.2 [checkered bar] and β-actin accumulation in comparison to controls. [F–H] Smn-deficient motoneurons exhibit reduced Ca2.2 and β-actin accumulation in comparison to controls. [I] The reduction of signal intensity for Ca2.2 and β-actin is restored by 100 μM 8-CPT-cAMP. 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.
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 motor endplate-specific forms of laminin (laminin-211/221). Previous papers have shown that neurite growth of motoneurons is impaired on this substrate (Porter et al., 1995). Furthermore, it has been shown that the β2 chain in laminin-211 interacts with the pore-forming (Caα) subunit of the N- and P/Q-type-specific VGCC (Ca,2.2 and Ca,2.1; Nishimune et al., 2004), indicating that presynaptic differentiation is mediated through the direct interaction of laminin-211 with Ca,2.2 channels. Smn-deficient motoneurons exhibit a reduced accumulation of Ca,2.2 channels in growth cones. This finding correlates with reduced Ca²⁺ influx and reduced spontaneous electrical activity in this part of the neuron.

Gene knockout mice for the lamin 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 motoneurone disease, as the defect can most probably be compensated by Cav2.2 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 postnatally 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...
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-Petruzelv, 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 ventralateral 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 U/ml penicillin G sodium and 50 U/ml streptomycin sulfate (Invitrogen), 827/1.50; 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 (Rossoll 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 g/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-HCl, 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 (Dianova). The Alto 647N–conjugated secondary antibody (Atto Technology) was used for STEED microscopy. Confocal images were obtained either with a microscope (TCS 4D; Leica) with a 100× oil-immersion objective (HCX PL APO CS), with identical settings for pinhole and voltage for control and experimental conditions, or with a microscope (SP2; Leica) with a 100× oil-immersion objective. An 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 (Rossoll 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
loaded with 2 μM FURA-2 AM (Invitrogen) at 37°C for 45 min in Ca²⁺ and Mg²⁺-containing HBSS. After washing, coverslips were mounted on a heated microscope stage [37°C] and constantly superfused with Ca²⁺/Mg²⁺-HBSS under linear flow conditions.

Measurements were performed by dual-excitation and dual-emission ratio imaging using a microscope fluorescence measurement workstation (Polychrome II, TILL Photonics). Mutant embryos were alternately exposed to monochromatic light of 340 and 380 nm, and emission was measured at 512 nm. Real-time microscope imaging was performed using a microscope (Axionview S100TV; Carl Zeiss MicroImaging, Inc.) equipped for epifluorescence with a dichroic mirror, a 100× 1.3 oil-immersion objective (Fluar; Carl Zeiss MicroImaging, Inc.), and a charge-coupled device camera (IMAGO; TILL Photonics).

Images were acquired at a frequency of 1 Hz and exposure time was 10 ms. The measurements in proximal axons were taken at a distance of 10–30 μm from the cell body. Spontaneous Ca²⁺ transients (340:380) were recorded separately in cell bodies, dendrites, axons, and growth cones over a time period of 7.5 min.

Permeabilizing the motoneurons with 20 μM digitonin allowed background signal detection after cytotoxic dye release for background correction. Cells were constantly superfused with Ca²⁺/Mg²⁺-HBSS over the registration time.

In situ hybridization
Hybridization solution containing 3 × biotinylated sense or antisense actin or N-type Ca²⁺ channel α1-glucuronides (200 ng/ml; GeneDetect) was applied to the coverslips. Hybridization was performed as described previously (Russoll et al., 2003). Images were acquired using a microscope (Axiophot; Carl Zeiss MicroImaging, Inc.) equipped with a charge-coupled device camera using Axioplan 2 software (Carl Zeiss MicroImaging, Inc.).

RT-PCR from neuronal stem cells
Total RNA from neuronal precursor cells was extracted by Trizol (Invitrogen) according to the manufacturer’s protocol, and 1 μg of total RNA was used for cDNA amplification. Amplification of the Smn2 cDNA was performed with Ex 5′ (5′-CCCTAATCTATCATGCT-3′) and Ex 8′ (5′-CTCAAC-ACCCCTTCTCAAGC-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).

Data analysis
Axons of motoneurons were identified by their length that processes that are at least two times longer than dendrites. Only the longest axonal branches were measured. Cultures obtained from mutant and control embryos from different litters were analyzed under a microscope (TCS4D; Leica), and axon length was measured from pictures using image software (Scion Corporation).

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 quantum 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 performed to correct for background signal detection after cytosolic dye release for background correction. Cells were constantly superfused with Ca²⁺/Mg²⁺-HBSS on the manuscript. We also thank Markus Dyba from Leica for support in STED microscopy.

We thank Christine Schneider for skillful technical assistance and Manfred Heckmann and Stefan Sigrist for many helpful suggestions and comments on the manuscript. We also thank Markus Dyba from Leica for support in STED microscopy.

This work was supported by grants from the Spinal Muscular Atrophy Foundation, the Deutsche Forschungsgemeinschaft (SFB 581, TP 81), and the Hermann and Lilly Schilling-Stiftung.

Submitted: 28 March 2007
Accepted: 11 September 2007

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