

Differentiation defects in primary motoneurons from a SMARD1 mouse model that are insensitive to treatment with low dose PEGylated IGF1

Frank Krieger¹, Friedrich Metzger², Sibylle Jablonka^{1,*}

¹Institute for Clinical Neurobiology; University of Wuerzburg; Wuerzburg, Germany; ²Roche Pharmaceutical Research and Early Development; Roche Innovation Center Basel; F. Hoffmann-La Roche Ltd.; Basel, Switzerland

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Abbreviations: Ighmbp2, immunoglobulin μ -binding protein 2; SMARD1, spinal muscular atrophy with respiratory distress type 1; SMA, spinal muscular atrophy; IGF1, insulin-like growth factor 1; Ca_v2.2, N-type specific calcium channel; PEG, polyethylene glycol

*Correspondence to: Sibylle Jablonka;
Email: Jablonka_S@ukw.de

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Muscle atrophy and diaphragmatic palsy are the clinical characteristics of spinal muscular atrophy with respiratory distress type 1 (SMARD1), and are well represented in the neuromuscular degeneration (*Nmd^{2J}*) mouse, modeling the juvenile form of SMARD1. Both in humans and mice mutations in the *IGHMBP2* gene lead to motoneuron degeneration. We could previously demonstrate that treatment with a polyethylene glycol-coupled variant of IGF1 (PEG-IGF1) improves motor functions accompanied by reduced fiber degeneration in the gastrocnemius muscle and the diaphragm, but has no beneficial effect on motoneuron survival. These data raised the question which cell autonomous disease mechanisms contribute to dysfunction and loss of Ighmbp2-deficient motoneurons. An analysis of primary Ighmbp2-deficient motoneurons exhibited differentiation deficits such as reduced spontaneous Ca²⁺ transients and altered axon elongation, which was not compensated by PEG-IGF1. This points to an IGF1 independent mechanism of motoneuron degeneration that deserves treatment approaches in addition to IGF1.

Introduction

Spinal muscular atrophy with respiratory distress type 1 (SMARD1) is a motoneuron disorder leading to early childhood death.^{1,2} The disease generally manifests in the first two years

after birth with a characteristic distal muscle atrophy due to motoneuron loss and paralysis of the diaphragm.^{1,3,4} As a monogenetic disorder, SMARD1 is caused by mutations in the *IGHMBP2* (*Immunoglobulin μ -binding protein 2*) gene on chromosome 11q13 which codes for an ATPase/helicase of the SF1 superfamily.^{1,5} The helicase domain is the hot-spot for most of the mutations.^{2,6} IGHMBP2 is predominantly localized in the cytoplasm of motoneurons where it is associated with ribosomes.^{6,7} The neuromuscular degeneration (*Nmd^{2J}*) mouse, an Ighmbp2-deficient mouse model, represents the juvenile form of SMARD1 and is characterized by weakness and atrophy of the gastrocnemius muscle beginning in the second to third week after birth, preceding a massive myopathy of the diaphragm.^{8,9} In contrast to some SMA mouse models, neuromuscular junctions (NMJs) of skeletal muscles partially remain intact during disease progression, and neurotransmission appears unaffected as long as NMJs are maintained.⁹ These observations raised the question whether and which cell autonomous disease mechanisms in motoneurons cause dysfunction and loss in consequence of Ighmbp2 deficiency. We could previously show that a systemic application of a polyethylene glycol-coupled IGF1 (PEG-IGF1)¹⁰ resulted in enhanced muscle strength accompanied by a significant improvement of muscle fiber calibers in *Nmd^{2J}* mice.¹¹ In addition, PEG-IGF1 supported axonal

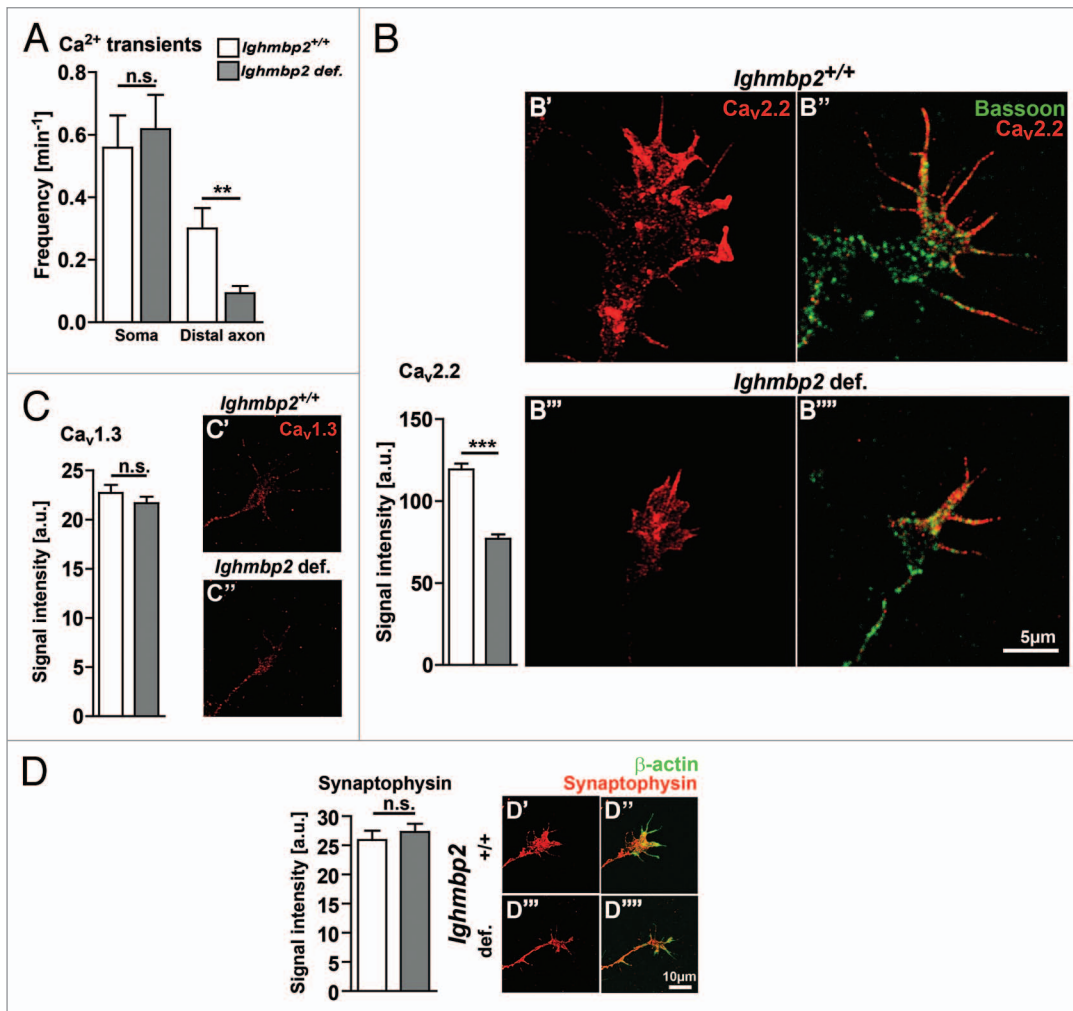


Figure 1. Disturbed calcium homeostasis at distal axons and altered $Ca_v2.2$ accumulation at growth cones of *Ighmbp2*-deficient motoneurons on the synapse-specific laminin-221. **(A)** Spontaneous Ca^{2+} transients in isolated *Ighmbp2*-deficient motoneurons were markedly reduced in the growth cone compartment (Controls $0.30 \pm 0.06 \text{ min}^{-1}$, $n = 22$, vs. *Ighmbp2* def. $0.09 \pm 0.02 \text{ min}^{-1}$, $n = 73$; $U = 487$, $p^{**} = 0.0047$). The Ca^{2+} transient frequency in the cell body of *Ighmbp2*-deficient motoneurons was not affected (Controls $0.56 \pm 0.10 \text{ min}^{-1}$, $n = 116$, vs. *Ighmbp2* def. $0.62 \pm 0.11 \text{ min}^{-1}$, $n = 45$; $U = 2,152$, $P = 0.08$). **(B)** The quantitative analysis of $Ca_v2.2$ presence in *Ighmbp2*-deficient growth cones revealed a significant reduction (Controls $119.3 \pm 3.5 \text{ a.u.}$, $n = 92$, vs. *Ighmbp2* def. $77.1 \pm 2.6 \text{ a.u.}$, $n = 106$; $U = 1,622$, $p^{***} < 0.001$). In **(B', B''')** a decreased $Ca_v2.2$ accumulation in the protrusions of *Ighmbp2*-deficient growth cones is displayed, co-labeled with the active zone marker Bassoon **(B'', B''')**. Analysis of $Ca_v1.3$ channels showed neither an altered distribution **(C', C'')** nor reduced levels **(C)** in *Ighmbp2*-deficient motoneurons (Controls $22.7 \pm 0.8 \text{ a.u.}$, $n = 76$, vs. *Ighmbp2* def. $21.7 \pm 0.7 \text{ a.u.}$, $n = 81$; $U = 3,000$, $P = 0.78$). **(D)** No difference in signal intensity of synaptophysin was detected between *Ighmbp2*-deficient and control motoneurons (Controls $25.9 \pm 1.6 \text{ a.u.}$, $n = 90$, vs. *Ighmbp2* def. $27.3 \pm 1.4 \text{ a.u.}$, $n = 90$; $U = 3,552$, $P = 0.15$). Synaptophysin was evenly distributed throughout the growth cone compartment and exhibited no difference regarding localization between *Ighmbp2*-deficient **(D''', D''')** and control motoneurons **(D', D'')**. Single values were obtained from at least three different experiments. Statistical analysis was performed using Mann-Whitney t test. Bars represent mean \pm SEM, significance is indicated by stars ($p^{**} < 0.01$, $p^{***} < 0.001$, non-significance is indicated by n.s.).

sprouting at the nerve terminals of the gastrocnemius muscle leading to reduced denervation activity.¹¹ Although PEG-IGF1 stimulated the phosphorylation of the ribosomal protein S6 kinase in spinal cord of *Nmd2^{fl}* mice,¹¹ the treatment did not increase motoneuron survival as recently reported for the progressive motoneuronopathy (*pnn*) mouse model.¹² These observations led to the hypothesis that *Ighmbp2*-deficient motoneurons

may not be amenable to PEG-IGF1, and survival is a cell autonomous disease mechanism caused by *Ighmbp2* deficiency, which can't be compensated by enhanced protein S6 kinase signaling. In order to investigate which specific disease mechanisms are caused by *Ighmbp2* deficiency in motoneurons, we performed a morphological and functional analysis of isolated primary motoneurons from *Ighmbp2*-deficient

embryonic spinal cord and investigated the effect of PEG-IGF1.

Reduced Frequency of Spontaneous Ca^{2+} Transients in *Ighmbp2*-Deficient Motoneurons

The complex phenotype of the *Nmd2^{fl}* mouse with atrophy of the gastrocnemius

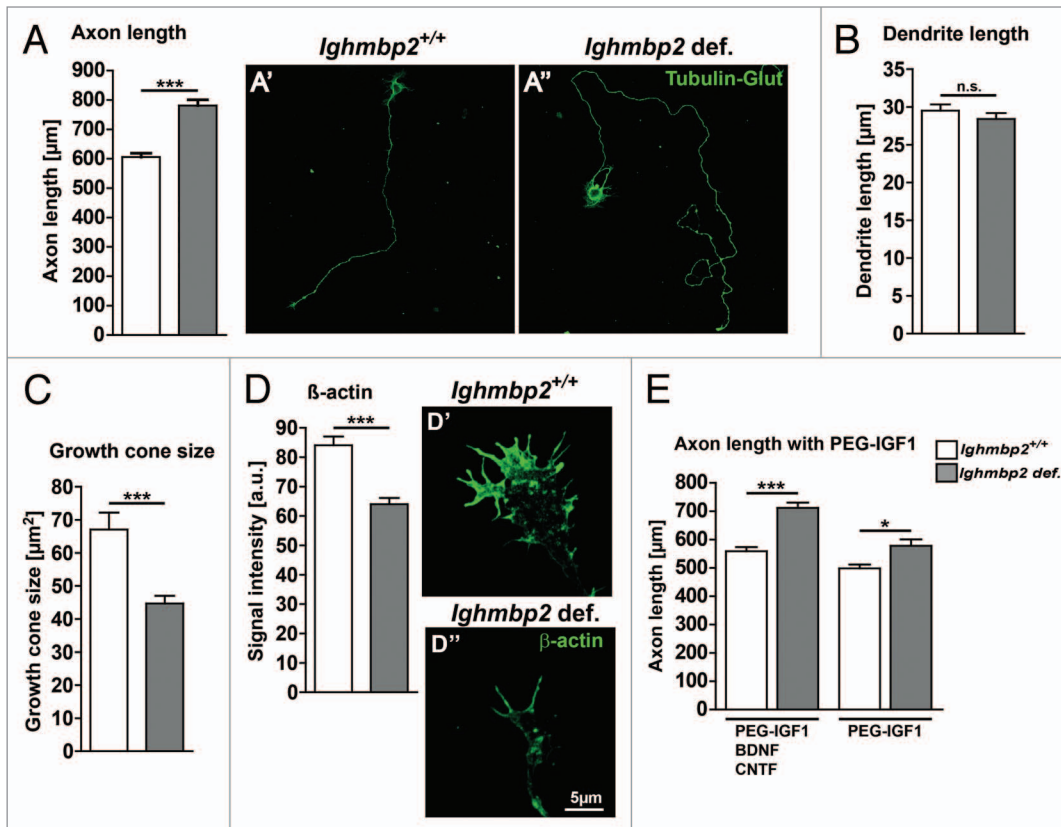


Figure 2. Low doses of PEG-IGF1 do not compensate for the axon elongation defect in *Ighmbp2*-deficient motoneurons on the synapse-specific laminin-221. In (A-C) the quantitative analyses of axon length, dendrite length, and growth cone size of *Ighmbp2*-deficient and control motoneurons are shown. (A) Isolated *Ighmbp2*-deficient motoneurons exhibited an altered axonal processing on laminin-221 (Controls $605.7 \pm 13.4 \mu\text{m}$, $n = 585$, vs. *Ighmbp2* def. $780.9 \pm 19.8 \mu\text{m}$, $n = 492$; $U = 104,000$, $p^{***} < 0.001$). Representative images are shown in (A', A''). (B) The dendrite length of *Ighmbp2*-deficient motoneurons was comparable to the control situation (Controls $28.4 \pm 0.8 \mu\text{m}$, $n = 292$, vs. *Ighmbp2* def. $28.4 \pm 0.8 \mu\text{m}$, $n = 206$; $U = 30,010$, $P = 0.97$). In (C) a markedly growth cone size reduction is depicted (Controls $67.0 \pm 5.2 \mu\text{m}^2$, $n = 78$, vs. *Ighmbp2* def. $44.7 \pm 2.4 \mu\text{m}^2$, $n = 102$; $U = 2,739$, $p^{***} = 0.0004$). (D) The β -actin distribution and localization was disturbed in growth cones of *Ighmbp2*-deficient motoneurons (Controls 84.0 ± 3.0 a.u., $n = 105$, vs. *Ighmbp2* def. 64.0 ± 2.1 a.u., $n = 107$; $U = 3,486$, $p^{***} < 0.001$). Representative images are shown in (D', D''). In (E) the quantification of axon lengths from control and *Ighmbp2*-deficient motoneurons in the presence of CNTF (C), BDNF (B) (10 ng/ml, each), and PEG-IGF1 (I) (1 ng/ml), and PEG-IGF1 (I) (1 ng/ml) alone is depicted (C+B+I: Controls $558.9 \pm 14.6 \mu\text{m}$, $n = 375$, vs. *Ighmbp2* def. $711.7 \pm 18.8 \mu\text{m}$, $n = 402$; $U = 55,050$, $p^{***} < 0.001$. I: Controls $498.4 \pm 13.6 \mu\text{m}$, $n = 301$, vs. *Ighmbp2* def. $577.7 \pm 22.7 \mu\text{m}$, $n = 190$; $U = 25,090$, $p^* = 0.02$). Single values were obtained from at least three different experiments. Statistical analysis was performed using Mann-Whitney t test. Bars represent mean \pm SEM, significance is indicated by stars ($p < 0.05$, $p^{***} < 0.001$, non-significance is indicated by n.s.).

muscle and myopathy of the diaphragm poses the question of which cell autonomous disease mechanisms could correspond to their dysfunction. We have already shown that isolated SMA type I mouse motoneurons are characterized by reduced spontaneous Ca^{2+} transients caused by defective clustering of N-type-specific Ca^{2+} channels ($\text{Ca}_v2.2$), that results in altered axon elongation on the synapse-specific laminin-221.¹³ In order to examine affected cellular mechanisms under *Ighmbp2* deficiency in more detail, we started a similar in vitro approach with isolated and enriched mouse motoneurons. Motoneurons from two week-old

Ighmbp2-deficient and control embryos were cultured for five days in the presence of BDNF and CNTF (10 ng/ml, each) on laminin-221 to quantify the frequency of spontaneous Ca^{2+} transients via imaging studies. For the calcium imaging study cells were loaded with FURA-2, a ratiometric Ca^{2+} -binding fluorescent dye. Measurements were performed by dual-excitation (340 and 380 nm) and single-emission ratio imaging. Images were acquired at a frequency of 1 Hz and exposure time was 10 ms. Spontaneous Ca^{2+} transients were recorded in growth cones and cell bodies over a time period of 5 to 7.5 min. The rate of spontaneous

Ca^{2+} transients (events per min) in the cell body of *Ighmbp2*-deficient motoneurons was not changed, whereas in the axon terminals spontaneous Ca^{2+} elevations decreased markedly (Fig. 1A). We further investigated the distribution of $\text{Ca}_v2.2$ and $\text{Ca}_v1.3$ channels in the growth cone of control and *Ighmbp2*-deficient motoneurons. Anti- $\text{Ca}_v2.2$ stainings revealed a reduced accumulation of $\text{Ca}_v2.2$ at the growth cone of *Ighmbp2*-deficient motoneurons compared with control motoneurons (Fig. 1B' and 1B''). Membrane-localization was indicated by co-staining with Bassoon (Fig. 1, 1B'', and 1B'''). Immunostaining for

Ca_v1.3 that normally does not cluster in active zones in vivo¹⁴ exhibited no altered distribution or reduced signal intensity (Fig. 1C, 1C', and 1C''), indicating that the defect is specific for Ca_v2.2 in axon terminals of *Ighmbp2*-deficient motoneurons. Reduced excitation in cultured motoneurons together with affected signal transduction of action potentials along the motor axons in *Nmdp1* mice⁹ raised the question whether synaptic vesicles are also reduced in the presynaptic compartment of *Ighmbp2*-deficient primary motoneurons. We did not detect any difference concerning presence and distribution of synaptophysin immunoreactivity between isolated *Ighmbp2*-deficient and control motoneurons (Fig. 1D and 1D'–1D'').

Low Doses of PEG-IGF1 do not Compensate for Altered Axon Elongation of *Ighmbp2*-Deficient Motoneurons on laminin-221

Based on our calcium imaging results and on our data from *Smn*-deficient motoneurons¹³ we performed a morphological analysis of the differentiation behavior of *Ighmbp2*-deficient motoneurons. Wild-type and *Ighmbp2*-deficient motoneurons were cultured on laminin-221 in the presence of CNTF and BDNF (10 ng/ml, each) and differentiation was analyzed by quantification of axon and dendrite lengths at DIV7, and growth cone sizes at DIV5. *Ighmbp2*-deficient mouse motoneurons showed enhanced axon elongation (Fig. 2A, 2A', and 2A''), whereas the dendrite length was unaffected (Fig. 2B). In addition to the altered axonal outgrowth, we also detected significant smaller growth cones corresponding to a microfilament deficit indicated by reduced β -actin presence in the growth cones of *Ighmbp2*-deficient motoneurons (Fig. 2C, 2D, 2D', and 2D''). Application of PEG-IGF1 (1 ng/ml) to the medium during the whole culture period did not modify the enhanced axon elongation of *Ighmbp2* deficient motoneurons on laminin-221 (Fig. 2E). We have chosen 1 ng/ml PEG-IGF1 for this long-term treatment as the optimal survival effect for

one-week cultures of isolated motoneurons lies within the range of 1 ng/ml and 10 ng/ml (data not shown).

Cell Autonomous Disease Mechanisms Observed in Primary Motoneuron Cultures Differ between Mouse Models for Motoneuron Disorders

Prolonged axonal elongation on laminin-221 of *Ighmbp2*-deficient motoneurons due to reduced frequency of Ca²⁺ transients reflects nerve cell autonomous disease mechanisms at a presymptomatic stage, similar to the results observed in studies with motoneurons deficient for *Smn* and its interaction partner hnRNPR, in mouse and zebrafish.^{13,15} Such kind of defects in axon elongation, corresponding to excitation disturbances are specific and are not seen in all mouse models for motoneuron diseases. They are observed in SMA and SMARD1 motoneurons, but e.g. not in isolated motoneurons from *SOD1*^{G93A} mutant mice, a model for a familial form of amyotrophic lateral sclerosis.¹⁶ However, the *pnn* mouse is another model with differentiation defects in primary motoneurons. But the disease mechanisms differ insofar that reduced axon lengths on laminin-111 due to a defective microtubule assembly in *pnn* motoneurons can be fully compensated by external application of PEG-IGF1.¹² Not only in cell culture, also in vivo, PEG-IGF1 prevents *pnn* motoneurons from degeneration.¹² These data ultimately argue for specific cell autonomous disease mechanisms corresponding to particular genetic dysfunctions in each mouse model.

Isolated motoneurons even from wild-type mouse embryos behave differently under specific culture conditions (data not shown). Neurotrophic factors and matrix proteins are only two components that could influence the axonal outgrowth and the excitation of cultured primary motoneurons. As we already demonstrated for isolated motoneurons from a SMA type I mouse model, axonal extension and local excitation depend, among others, on the matrix protein.¹³ Thus one can't

draw conclusions to the axon elongation behavior in vivo from morphological and even functional studies with primary motoneurons. The influence of the neighboring non-neuronal tissue and the fast changing developmental stages during motoneuron maturation in vivo is far too high. However, differentiation behavior of enriched primary murine motoneurons from motoneuron disease models are a very important technical tool to understand altered cellular mechanisms, especially signaling pathways that might affect motoneuron development at very early symptomatic or even pre-symptomatic stages. Enriched primary motoneurons, albeit they do not completely represent the in vivo situation, are useful to investigate cellular dysfunctions in more detail. The more we know about cell-specific dysregulations at very early disease stages, the higher the possibility to identify the mechanisms that could bypass the affected cellular targets in the face of potential therapeutic strategies.

PEG-IGF1 Induced Akt/protein S6 Kinase Pathway Activation has no Compensatory Effect in Motoneurons when *Ighmbp2* is Missing

As it is currently known, PEG-IGF1 stimulates the Akt/protein S6 kinase phosphorylation in isolated motoneurons and in spinal cord tissue from *Nmdp1* mice, but does not prevent motoneuron degeneration in vivo¹¹ and differentiation defects in vitro (Fig. 2E). This is in contrast to the observed positive effects in striated muscles of *Nmdp1* mice. Four-week treatment with 0.05 mg/kg PEG-IGF1 delayed muscle fiber degeneration, both in the gastrocnemius muscle and the diaphragm.¹¹ One reason might be the “underdosed” treatment, as already discussed by Krieger et al.¹¹ as doses higher than 0.05 mg/kg were not tolerated by the *Nmdp1* mouse. Higher doses were needed to obtain a beneficial effect on motoneuron survival in other mouse models for motoneuron degeneration such as the *pnn* mouse.¹² However, with regard to our in vitro data, it is even

more likely that Ighmbp2 deficiency places a gap in the IGF1 receptor/Akt/p70^{S6K} signaling pathway. p70^{S6K} is discussed to be an important factor for translation initiation.¹⁷ The ability for Akt/p70^{S6K} stimulation in embryonic motoneurons indicates that Ighmbp2 is acting downstream of the p70^{S6K} signaling pathway or acts completely independent from the Akt/mTOR/p70^{S6K} pathway. Ighmbp2 is a ribosome associated ATPase/helicase mainly localized to the cytoplasm and subjected to unwinding RNAs.⁶ The specific role of Ighmbp2 remains to be elucidated, but possible scenarios could be discussed as follows: (1) Ighmbp2 assists the proper orchestration of ribosome-assembly, (2) the helicase could be involved in the regulation of tRNA pathways as already postulated,⁷ or (3) Ighmbp2 plays a role in translation initiation via processing of mRNAs by unwinding secondary structures, and/or removing microRNAs or bound proteins. Especially the defective translation of mRNAs that encode for proteins with functions for axon maintenance, such as axonal transport or transmission of action potentials along motor axons, could cause motoneuron and motor axon degeneration. Axonally transported mRNAs are, e.g., those that encode for cytoskeletal proteins such as Tau^{18,19} and neurofilament.²⁰ Proteins that are important for action potential transmission are, among others, sodium and potassium channels.

Excitation in Motoneurons Affected by Ighmbp2 Deficiency

Developing motoneurons in vertebrates exhibit spontaneous calcium elevations while they are targeting striated muscles.²¹⁻²³ In isolated embryonic motoneurons from rodents, spontaneous excitation is preserved and observed in axons and axonal growth cones.^{13,24} Spontaneous Ca²⁺ transients in neurons contribute to presynaptic differentiation via interaction with matrix proteins.^{13,25,26} Numerous publications have already reported the impact of Smn deficiency on excitation and Ca²⁺ gating channels in neurons and glia cells from mouse models

of proximal spinal muscular atrophy (SMA).^{13,24,27-30} As we described herein, spontaneous excitation disturbances were also observed in Ighmbp2-deficient motoneurons (Fig. 1A) which led to axonal elongation defects (Fig. 2A). However, not only in vitro but also in vivo, variabilities in excitation were monitored.⁹ Electrophysiological recordings at the affected LAL muscle of *Nmd^{2J}* mice showed a higher probability for “failures,” stimuli that failed to evoke postsynaptic responses during nerve repetitive stimulation, but with preserved EPP amplitudes.⁹ We, therefore, conclude from these findings that the failures were not due to a defect in synaptic vesicle availability, in Ca²⁺ entry, or in the release machinery, and that axonal degeneration is hence independent from those neurotransmission impairments.⁹ This encouraged us to postulate that defects or deficits of ion channels, such as potassium or sodium channels, may also have an impact on motor axon dysfunction and degeneration. As already reported, a member of the voltage gated sodium channel family termed Na_v1.9 supports differentiation in early motoneurons via axonal excitation.^{24,31} We postulate that Na_v1.9 in the adult mouse has an impact on rapid propagation of action potentials along the axon, and that a deficit or defect of this channel might impair axonal stability independent from neurotransmission. The detailed analysis on localization and function of Na_v1.9 in the *Nmd^{2J}* mouse would reveal first hints whether sodium channels are affected targets in an Ighmbp2-deficient environment.

Affected Microfilaments in Ighmbp2-Deficient Motoneurons

The observed excitability disturbances in vitro and in vivo⁹ could be a primary or secondary effect due to Ighmbp2 deficiency. As discussed and postulated by others, Ighmbp2/IGHMBP2 could play a pivotal role in cellular protein biosynthesis mechanisms.^{6,7} We already know from our studies that the IGF1/Akt/p70^{S6K} pathway is still inducible in Ighmbp2-deficient motoneurons,¹¹ which draw the attention to other signaling pathways important

for the microfilament orchestration. We observed a reduction in β-actin protein, suggesting that the translation of this mRNA is affected when Ighmbp2 is missing. As local protein biosynthesis in different cellular compartments plays a role especially in axon guidance mechanisms,³² Ighmbp2 deficiency might diminish the recognition of guidance cue signaling. Shigeoka and Holt have summarized known guidance cues and their target mRNAs.³² β-actin mRNA is a very prominent target candidate for local protein synthesis, as Netrin1³³⁻³⁷ and the neurotrophic factors BDNF and NGF^{38,39} stimulate its local translation. These results are obtained from retinal ganglia cells, hippocampal, cortical, and spinal neurons. The wide abundance of actin mRNA in neurons and the indispensability of microfilaments in the orchestration of different cellular compartments, especially the proper localization of transmembrane proteins such as receptors or ion channels, arises the hypothesis that Ighmbp2-deficient motoneurons do not correctly recognize and/or process signaling mediated by guidance cues or neurotrophic factors. This disability could affect motoneuron survival, differentiation, proper reaching of the target tissue, and subsequent maturation. Insufficient maturation of neuromuscular endplates has been already described in the mouse model for SMARD1,^{9,40} thus pointing to a mechanism which affects neuromuscular maturation, but might also compromise other cellular compartments.

Conclusion

Our in vitro data showed that Ighmbp2 deficiency causes altered cell autonomous mechanisms which lead to affected differentiation behavior. The application of 1 ng/ml of PEG-IGF1 had no modifying effect on these alterations. These results underline our in vivo data from the *Nmd^{2J}* mouse.¹¹ Four-week treatment with PEG-IGF1 activates the Akt/p70^{S6K} pathway, but does not result in increased motoneuron survival.¹¹ This raises the question of which cellular targets are affected by Ighmbp2 deficiency. Beside

the identification of targeted RNAs and their proteins in *Ighmbp2*-deficient motoneurons, the specific examination of other survival and differentiation supporting mechanisms, such as protein biosynthesis or RNA/protein degradation, would highly improve the understanding of motoneuron degeneration in *SMARD1*. The analysis of affected and unaffected

cellular mechanisms will facilitate the identification of signaling pathways, which compared with IGF1 signaling better bypass cellular dysfunctions caused by *Ighmbp2* deficiency.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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