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

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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 (Nmd2J) 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 Ca\textsuperscript{2+} 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.\textsuperscript{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.\textsuperscript{1,3,4} As a monogenetic disorder, SMARD1 is caused by mutations in the IGHMBP2 (Immunoglobulin \(\mu\)-binding protein 2) gene on chromosome 11q13 which codes for an ATPase/helicase of the SF1 superfamily.\textsuperscript{1,5} The helicase domain is the hot-spot for most of the mutations.\textsuperscript{2,6} IGHMBP2 is predominantly localized in the cytoplasm of motoneurons where it is associated with ribosomes.\textsuperscript{6,7} The neuromuscular degeneration (Nmd2J) 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.\textsuperscript{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.\textsuperscript{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)\textsuperscript{10} resulted in enhanced muscle strength accompanied by a significant improvement of muscle fiber calibers in Nmd2J mice.\textsuperscript{11} In addition, PEG-IGF1 supported axonal

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

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sprouting at the nerve terminals of the gastrocnemius muscle leading to reduced denervation activity. Although PEG-IGF1 stimulated the phosphorylation of the ribosomal protein S6 kinase in spinal cord of Nmd2fl mice, the treatment did not increase motoneuron survival as recently reported for the progressive motoneuronopathy (pmn) mouse model. 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 Ca²⁺ Transients in Ighmbp2-Deficient Motoneurons**

The complex phenotype of the Nmd2fl mouse with atrophy of the gastrocnemius...
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\textsuperscript{2+} transients caused by defective clustering of N-type-specific Ca\textsuperscript{2+} channels (Ca\textsubscript{v2.2}), that results in altered axon elongation on the synapse-specific laminin-221. 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\textsuperscript{2+} transients via imaging studies. For the calcium imaging study cells were loaded with FURA-2, a ratiometric Ca\textsuperscript{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\textsuperscript{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\textsuperscript{2+} transients (events per min) in the cell body of Ighmbp2-deficient motoneurons was not changed, whereas in the axon terminals spontaneous Ca\textsuperscript{2+} elevations decreased markedly (Fig. 1A). We further investigated the distribution of Ca\textsubscript{v2.2} and Ca\textsubscript{v1.3} channels in the growth cone of control and Ighmbp2-deficient motoneurons. Anti-Cav2.2 stainings revealed a reduced accumulation of Cav2.2 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\textsuperscript{2+} transients (events per min) in the cell body of Ighmbp2-deficient motoneurons was not changed, whereas in the axon terminals spontaneous Ca\textsuperscript{2+} elevations decreased markedly (Fig. 1A). We further investigated the distribution of Ca\textsubscript{v2.2} and Ca\textsubscript{v1.3} channels in the growth cone of control and Ighmbp2-deficient motoneurons. Anti-Ca\textsubscript{v2.2} stainings revealed a reduced accumulation of Ca\textsubscript{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\textsuperscript{2+} transients (events per min) in the cell body of Ighmbp2-deficient motoneurons was not changed, whereas in the axon terminals spontaneous Ca\textsuperscript{2+} elevations decreased markedly (Fig. 1A). We further investigated the distribution of Ca\textsubscript{v2.2} and Ca\textsubscript{v1.3} channels in the growth cone of control and Ighmbp2-deficient motoneurons. Anti-Ca\textsubscript{v2.2} stainings revealed a reduced accumulation of Ca\textsubscript{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’’”).
Ca₁.₃ 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 Ca₂⁺ 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 Nmd²⁻ mice¹⁰ 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 Autonomic 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 SOD1²⁻ 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 Nmd²⁻ 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 Nmd²⁻ 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 Nmd²⁻ 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 IGFI receptor/Akt/p70S6K signaling pathway. p70S6K is discussed to be an important factor for translation initiation. 17 The ability for Akt/p70S6K stimulation in embryonic motoneurons indicates that Ighmbp2 is acting downstream of the p70S6K signaling pathway or acts completely independent from the Akt/mTOR/p70S6K 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 8,19 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. 15,24 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 Nmd2J 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 Na1.9 supports differentiation in early motoneurons via axonal excitation. 24,31 We postulate that Na1.9 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 Na1.9 in the Nmd2J 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 vivo 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 IGFI/Akt/p70S6K 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. 33 β-actin mRNA is a very prominent target candidate for local protein synthesis, as Netrin1 34-37 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 Nmd2J mouse. 11 Four-week treatment with PEG-IGF1 activates the Akt/p70S6K 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 Igelmhp2-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 Igelmhp2 deficiency.

Disclosure of Potential Conflicts of Interest
No potential conflict of interest was disclosed.


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