The SMA Modifier Plastin 3 Targets Cell Membrane-Associated Proteins in Motoneurons

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ABSTRACT: Loss of the *Survival Motor Neuron (SMN)* gene inevitably leads to spinal muscular atrophy (SMA), one of the most common fatal neuromuscular diseases in children with FDA and EMA approved therapies. However, the cellular mechanisms leading to neuromuscular junction (NMJ) dysfunction due to impaired Ca^{2+} homeostasis in the presynaptic compartment remain largely unexplained. In the last decade, the so-called SMA modifiers have gained attention. The F-actin bundler Plastin 3 (PLS3) is one of them and counteracts neurotransmission defects, including altered vesicle endocytosis, in Smn-deficient NMJs. Properly bundled F-actin is the basis for the translocation and arrangement of transmembrane proteins at the cell surface. Our recently published data by Hennlein et al., J Cell Biol. (2023) clearly showed that Smn deficiency impairs the F-actin dependent translocation of the high-affinity BDNF receptor TrkB to the cell surface resulting in reduced BDNF-mediated TrkB activation in motor axon terminals. Strikingly, the overexpression of PLS3 restores TrkB availability, and significantly improves the clustering of the active zone-associated voltage-gated calcium channel $Ca_v2.2$ in growth cones of Smn-deficient motoneurons. These observations raise the question of how PLS3 mediates the proper cell surface localization and cluster-like formation of $Ca_v2.2$ in motor axon terminals.

KEYWORDS: Plastin 3, Cacna2d2, 3D cell culture, $Ca_v2.2$, BDNF/TrkB, spinal muscular atrophy, motoneuron

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Our study by Hennlein et al., J Cell Biol. (2023) demonstrated that in Smn-deficient motoneurons Plastin 3 (PLS3) modifies F-actin dependent cell surface translocation of TrkB and the cluster-like formation of $Ca_v2.2$, thereby regulating local Ca^{2+} homeostasis in motor axons.[1]

In this commentary, we will (1) summarize the observations on PLS3-mediated cell surface translocation of the high-affinity BDNF receptor TrkB and the cluster-like formation of the active zone-associated voltage-gated calcium channel (VGCC) $Ca_v2.2$, and (2) discuss possible mechanisms by which Plastin 3 deficiency might affect $Ca_v2.2$ clustering in motor axon terminals. In addition, we will present a spinal cord tissue model that can be used to study motoneuron diseases in 3D to further elucidate previously unrecognized cellular pathomechanisms.

The study provides evidence that TrkB phosphorylation after BDNF stimulation is reduced in Smn-deficient motoneurons although TrkB presentation at the cell surface is preserved. From previous work, we know that the disruption of TrkB in mice causes differentiation defects in motoneurons.[2] Neurotrophic factor signaling activates several signaling pathways that are essential for the survival and differentiation of motoneurons. It was therefore striking to observe that in TrkB kinase domain-deficient conditions, morphological and functional changes such as impaired motor axon elongation and the corresponding changes in spontaneous calcium transients,[2] phenocopy those observed in SMA motoneurons.[3,4] Inevitably, this raised the question of the extend to which BDNF/TrkB signaling is altered in SMA. We were able to show that BDNF-mediated endocytosis of TrkB is not impaired, but activity-mediated TrkB recruitment to the cell surface and its re-translocation after BDNF-mediated activation is reduced. After BDNF stimulation, TrkB levels are restored up to 67% in control cells, but only to 40% in Smn-deficient cells. We concluded that a local self-amplifying mechanism of BDNF affects the availability of TrkB at the cell membrane in an F-actin cytoskeleton-dependent manner.[1] From our previous studies we know that primary motoneurons from SMA type I mouse models have reduced levels of β-actin, resulting in reduced growth cone size and impaired axon elongation.[3,4] F-actin dynamics depend on F-actin bundling proteins that associate with filopodia to reinforce the protrusive actin network. One candidate for such an F-actin bundling protein is PLS3,[5,6] a so-called SMA modifier. Transcriptome-wide differential expression analysis of *SMN1*-deleted siblings with discordant disease outcomes revealed a significant association between disease severity and PLS3 expression in female SMA patients.[7] PLS3 mRNA is reduced in the somatodentritic and axonal compartments of Smn-deficient primary cultured mouse motoneurons. This leads to a decreased velocity of F-actin movements in the growth cone which corresponds to a significant reduction in TrkB phosphorylation.[1] Conversely, hPLS3 overexpression normalizes the distribution of TrkB and its activity-dependent surface translocation in Smn-deficient motor axon terminals, which in turn restores BDNF-mediated TrkB phosphorylation and causes an increase in BDNF/TrkB

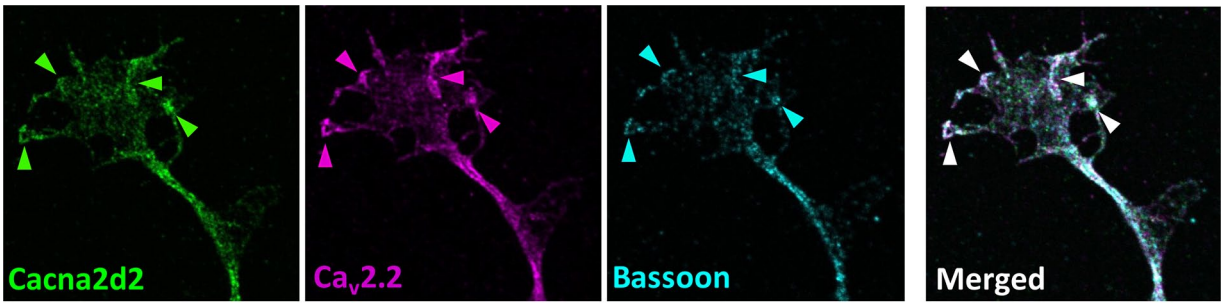

Figure 1. Cacna2d2 in growths cones of primary 2D-cultured mouse motoneurons. Cacna2d2 localizes to the axonal compartment and to the outer edge of growth cone protrusions together with $Ca_v2.2$ and Bassoon (arrowheads).

signaling.[1] These experiments suggest that PLS3 with its F-actin bundling properties is functionally involved in the dynamic surface presentation of TrkB and its activation by BDNF stimulation. In a previous study, we showed that the GDNF receptor c-Ret, also a tyrosine kinase receptor, as well localizes to the growth cone. However, in terms of axon elongation Smn-deficient motoneurons did not respond differently to GDNF than control motoneurons.[2] We found a distinct distribution of the GDNF receptor c-Ret and the BDNF receptor TrkB at the nerve terminals of embryonic motoneurons cultured on the β2-chain laminin-221. TrkB localizes to growth cone protrusions in close proximity to $Ca_v2.2$ calcium channels, whereas c-Ret is found in more central growth cone regions.[2] Thus, PLS3 may be important for transporting growth factor receptors and other transmembrane proteins along F-actin that are destined for localization at the outermost terminals of motor nerve endings to regulate axonal outgrowth/elongation.

Smn-deficient motoneurons suffer from an impaired frequency of spontaneous Ca^{2+} transients in the growth cones, which in turn corresponds to disturbed axon elongation.[3] Like TrkB, voltage-gated calcium channels are also located at the growth cone protrusions. Sh*PLS3* motoneurons show impaired F-actin movements in the growth cone, as evidenced by reduced distance traveled by individual filopodia and reduced velocity of the movement.[1] Based on this, we then hypothesized that the membrane translocation and especially the accumulation of VGCCs at presynaptic terminals might also be F-actin-dependent. High-resolution Structured Illumination Microscopy (SIM) revealed that the formation of $Ca_v2.2$ "cluster-like" accumulations is significantly reduced upon PLS3 knockdown, corresponding to a reduced spontaneous frequency of Ca^{2+} transients in the axon terminals similar to that observed in Smn-deficient motoneurons.[1] This led to the conclusion that PLS3 is required for the proper targeting and function of presynaptic transmembrane proteins such as the voltage-gated Ca^{2+} channel $Ca_v2.2$. However, the complexity of VGCC clustering raises the question of whether other proteins that are closely associated with Ca_v2 channels and dysregulated by Smn deficiency influence the assembly process. In a previous study, we have shown that the Cdk5 inhibitor R-Roscovitine, which has Ca^{2+} channel blocking properties and thus increases Ca^{2+} influx through the channel, alters the transcriptional profile of Smn-deficient motoneurons.[8] Therefore, to investigate the possibility that R-Roscovitine somehow affects the transcript levels of VGCCs in Smn-deficient motoneurons, an RNA-Seq approach was performed. Several transcripts were restored by R-Roscovitine on laminin-111, excluding those of N-type ($Ca_v2.2$) and P/Q-type ($Ca_v2.1$) calcium channels. In constrast, the auxiliary subunit α2δ2 (Cacna2d2) of Ca_v 2.1/2 was one of the candidates.[8]

The VGCC auxiliary subunits α2δ1-4 (Cacna2d1-4) are loosely associated with the VGCC complexes $Ca_v2.1/2$.[9] Recently, it has been reported that mutations in Cacna2d1 affect its cell surface localization and result in a complete inability of Cacna2d1 to enhance trafficking and function of Ca_v2 channels. This ultimately leads to the development of epileptic encephalopathy.[10] Contrary to the assumption that active zone scaffolding is Ca_v2-driven, it has been found that synapse and active zone assembly occurs in the absence of presynaptic Ca^{2+} channels and Ca^{2+} entry.[11] This raised the question of other candidates that might play a role in Ca^{2+} channel assembly which is dysregulated under Smn deficiency. The δ1-4 proteins are widely distributed in nerve terminals, and their localization does not depend on the presence of the Ca_v α1 subunit (Cacna1a). Some studies have discussed the auxiliary subunits of the N- and P/Q-type calcium channels as modulators of axon regeneration in dorsal root ganglia. Cacna2d2 has been described as a developmental switch that limits axon growth and regeneration, as *Cacna2d2* gene deletion or gene silencing promoted axon growth in vitro and pharmacological blockade of Cacna2d2 by administration of pregabalin (PGB) increased axon regeneration in adult mice after spinal cord injury.[12]

Our data provide strong evidence that PLS3 deficiency leads to impaired Ca^{2+} signaling and Ca^{2+} homeostasis in motor nerve terminals due to reduced $Ca_v2.2$ accumulation at the cell surface.[1] This raises the question whether PLS3 deficiency probably affects the cluster-like formation of $Ca_v2.2$ by disrupting the localization of Cacna2d2.

Since Cacna2d2 colocalizes with $Ca_v2.2$ in growth cones of primary 2D-cultured mouse motoneurons (Figure 1), it would be very interesting to find out if, where, and to what extent, Cacna2d2 plays a role in maintaining motoneuron function by modifying F-actin/PLS3-dependent $Ca_v2.2$ accumulations at

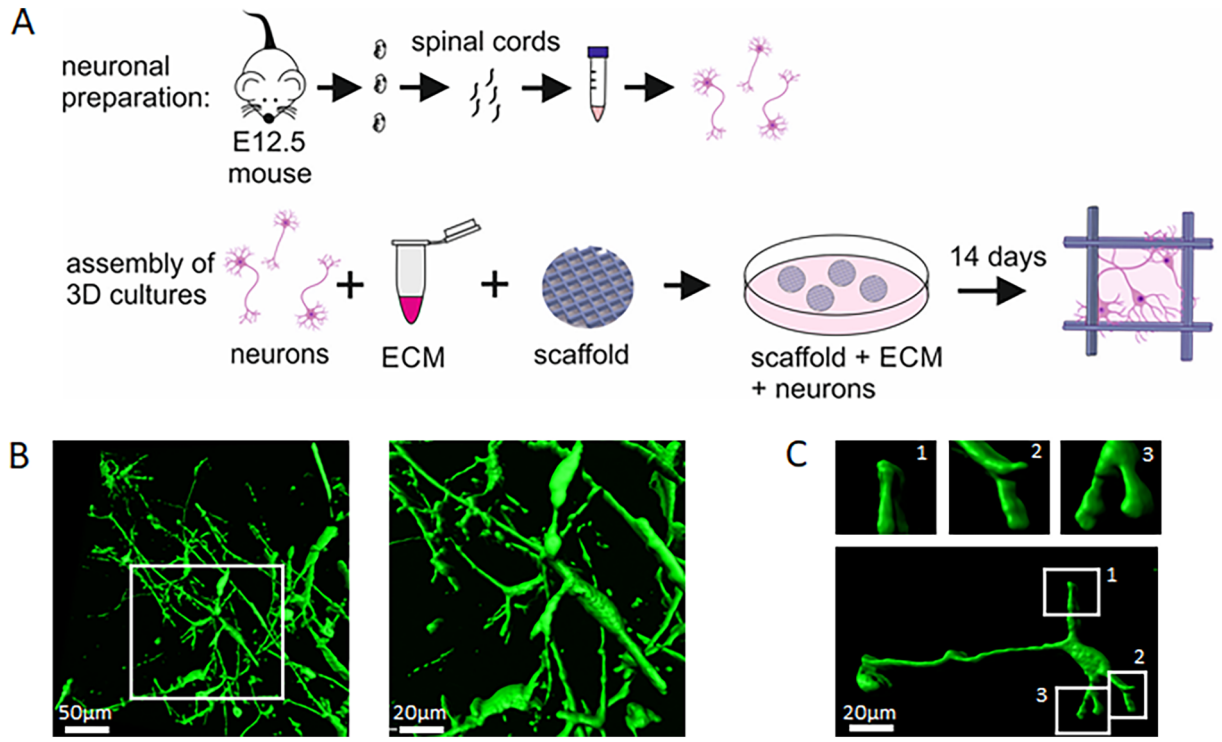

Figure 2. Preparation and imaging of 3D motoneuron cultures. (A) Isolation of motoneurons from E12.5 mouse embryos (upper lane). Organization of 3D spinal cord culture by combining primary neurons, ECM and a scaffold for reinforcement (lower lane) (modified from Fischhaber et al[14]). (B) Imaris reconstruction of a β-actin staining of a 4-day-old (DIV4) 3D motoneuron culture. (C) Single motoneuron with magnification of the nerve endings (1-3).

the cell surface of axonal growth cones. However, such studies would be best performed with high-resolution microscopy of 3D cell cultures, as the stiffness of 2D cell culture substrates contrasts with that of native neuronal tissue. Native tissue exhibits an ultrasoft, highly non-linear, and time-dependent material behavior.[13] In addition, cells within the nervous system respond to their mechanical environment which is critical for tissue maturation and plays an important role in disease progression, injury, and regeneration, and is not present in commonly used 2D cultures. Figure 2 illustrates a previously established and studied 3D spinal cord model system.[14] Printed 3D scaffolds have been used to mechanically support weak extracellular matrix (ECM) formulations that mimic the soft CNS and are particularly suitable for the peripheral nervous system. These reinforcements are needed to ensure sample stability throughout the analysis period and during experiments. Instead of scaffolds, printed nanofibers can also be used as reinforcing structures.[15] In a second step primary spinal cord neurons or pure motoneuron cultures are prepared, counted, mixed with the ECM and then pipetted onto the scaffolds or fibers (Figure 2A). After days or weeks in culture, the neuronal network is mature and can be used for further analysis (Figure 2B and C). Initial studies in wild-type 3D spinal cord and cortical cultures have shown that neuronal networks grow faster in 3D and show significant differences compared to age-matched 2D cultures, for example, in synaptic density or dendritic length.[14,16] These differences in morphology and protein expression, in terms of the correct arrangement of proteins in the different cell compartments, can be visualized more precisely by 3D cultures.

Combined with the use of high-resolution microscopy techniques and appropriate analysis software to generate and analyze 3D reconstructions, these 3D cultures could elucidate pathophysiological mechanisms that were previously invisible in a 2D cell culture system. In the long term this could lead to far more better insights into molecular and cellular structures to better understand the progression of cellular pathomechanisms.

Conclusion

Our study on the importance of PLS3 in the formation of $Ca_v2.2$ cluster-like structures suggests a possible role for the auxiliary subunit Cacna2d2 (α2δ2) in this mechanism. However, in order to draw further conclusions on the effect of Cacna2d2 on the localization and function of $Ca_v2.2$ in motor axons, it is necessary to use appropriate cell culture systems. Only with a 3D culture of motoneurons in combination with high-resolution microscopy would it be technically possible to visualize such reciprocal functional dependencies to get an approximate idea of the modifying function of auxiliary subunits such as Cacna2d2 (α2δ2) on the accumulation of active zone-associated voltage-gated Ca^{2+} channels also in SMA and other motoneuron diseases.

Author Contributions

SJ conceived the manuscript and then SJ and NS wrote and revised the manuscript.

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