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Cell-autonomous axon growth of young motoneurons is triggered by a voltage-gated sodium channel

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Spontaneous electrical activity preceding synapse formation contributes to the precise regulation of neuronal development. Examining the origins of spontaneous activity revealed roles for neurotransmitters that depolarize neurons and activate ion channels. Recently, we identified a new molecular mechanism underlying fluctuations in spontaneous neuronal excitability. We found that embryonic motoneurons with a genetic loss of the low-threshold sodium channel $Na_V1.9$ show fewer fluctuations in intracellular calcium in axonal compartments and growth cones than wild-type littermates. As a consequence, axon growth of $Na_V1.9$-deficient motoneurons in cell culture is drastically reduced while dendritic growth and cell survival are not affected. Interestingly, $Na_V1.9$ function is observed under conditions that would hardly allow a ligand- or neurotransmitter-dependent depolarization. Thus, $Na_V1.9$ may serve as a cell-autonomous trigger for neuronal excitation. In this addendum, we discuss a model for the interplay between cell-autonomous local neuronal activity and local cytoskeleton dynamics in growth cone function.

Keywords: motoneurons, sodium channel, axon growth, spontaneous excitation, $Na_V1.9$, local protein synthesis, spinal muscular atrophy

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In the developing nervous system, electrical activity is observed long before neurons are embedded in a synaptic network.[1,2] Early electrical activity is an evolutionarily conserved phenomenon and regulates a broad spectrum of developmental processes, such as cell proliferation, neuronal differentiation, cell migration, neuronal survival and neurite growth, as well as the refinement of synaptic connections.[2] Excitation of neurons before synapse formation is often termed as "spontaneous excitation" and in many cases the neuronal depolarization is accompanied by spontaneous influx of calcium ions to the neuronal cytosol. Investigating the molecular mechanisms underlying spontaneous calcium influx revealed two principle mechanisms of how spontaneous excitation is initiated. Either spontaneous excitation is ligand-dependent and caused by the non-synaptic release of transmitters such as glutamate or GABA; both act excitatory on immature neurons. Alternatively, excitability is part of a developmental program and thus a cell-autonomous feature of a young neuron. While there is an enormous progress in understanding the molecular mechanisms of ligand-dependent regulation of early electrical activity, the molecules regulating cell-autonomous excitability are barely defined.

In vertebrates, motoneurons exhibit spontaneous calcium transients while they are growing over long distances to the skeletal muscle.[2-4] When embryonic motoneurons from rodents are cultured at low density the phenomenon of spontaneous excitation is preserved and spontaneous calcium transients are preferentially observed in axons and axonal growth cones.[5,6] Spontaneous calcium transients in motoneurons contribute to axon elongation and presynaptic differentiation.[5] This is of pathophysiological relevance. Motoneurons isolated from a mouse model for the motoneuron disease spinal muscular atrophy generate less spontaneous calcium transients and this causes deficits in axonal growth and growth cone differentiation.[5]

Cell-Autonomous Excitability in Embryonic Motoneurons

Research aiming to decipher the pathophysiology of the motoneuron disease spinal muscular atrophy gave deeper insights in the molecular mechanisms and function underlying growth-mediating spontaneous excitability.[5,7,8]

Spinal muscular atrophy, commonly referred to as SMA, affects motoneurons in the anterior horn of the spinal cord and causes axonal defects and disturbed neuromuscular transmission, hence causing muscle weakness.[9-11] SMA is the most common genetic cause of infant mortality and is initiated by mutations in the Survival of Motor Neuron 1 (*SMN1*) gene.[10]

When embryonic motoneurons from a mouse model for a severe form of SMA (SMA type II) are analyzed in vitro, they show drastic changes in cell-autonomous functions. When these neurons are cultured on a laminin-isoform that supports axonal elongation, axon growth and growth cone size are reduced.[5,7] This coincides with lower levels of β-actin mRNA and β-actin protein in growth cones.[5,7] Recently, we confirmed that β-actin is locally synthesized in growth cones of motoneurons and this process is deregulated in SMN-deficient motoneurons.[12] Surprisingly, cytoskeletal defects in motoneuron growth cones cause defects in cell-surface clustering of the N-type calcium channel $Ca_V2.2$ and less local calcium influx events are observed.[5] Defects in the typical cell-autonomous pathology of SMN-deficient motoneurons in vitro can partly be rescued. When motoneurons are treated with a cell membrane permeable cAMP analog, distal actin mRNA and actin protein levels are increased, $Ca_V2.2$ levels are augmented in growth cones, and subsequently spontaneous calcium influx frequencies are normalized.[5] Thus, local translation of β-actin is most likely upstream of the mechanism for proper N-type channel clustering in distal axons.

Spontaneous calcium transients in motoneurons are observed in cells without synaptic contact, even under rapid perfusion with defined artificial cerebrospinal fluid solutions (**Fig. 1A** shows some examples, visualized at a slow timescale).[6] However, the N-type calcium channel $Ca_V2.2$ and the P/Q-type channel $Ca_V2.1$, both reside at presynaptic sites in motoneurons, normally require a strong depolarization for activation.[13] This predicts a trigger molecule with the following features: (1) expressed in motoneurons, (2) present in axons and growth cones, (3) low activation threshold, (4) spontaneous opening close to resting membrane potentials, (5) mediator of excitatory ion influx.

To characterize this trigger molecule was the aim of our study by Subramanian and Wetzel et al. (2012).[6] We investigated whether low threshold voltage-gated sodium channels mediate cell-autonomous excitability in young motoneurons.

As a first test, wild-typic motoneurons were cultured in the presence of low concentrations of pore blockers of voltage-gated sodium channels. Indeed, after several days in culture axonal elongation of motoneurons was reduced when motoneurons were treated with low amounts of saxitoxin (STX) and tetrodotoxin (TTX). Dendritic growth and motoneuron survival remained unaffected.

Voltage-gated sodium channels are key proteins for neuronal excitation and action potential initiation. However, as discussed above, spontaneous electrical activity in motoneurons is also a local event, and is therefore unlikely to be mediated by ligand-dependent initiation of action potentials. This raised the question which voltage-gated sodium channel is responsible for fluctuations in local excitability close to the resting membrane potential.

A Low-Threshold Voltage-Gated Sodium Channel Triggers Cell-Autonomous Excitation

The family of VGSCs comprises nine members, classified as $Na_V1.1$ to $Na_V1.9$.[14] In comparison to the other Na_V-channels, the TTX-insensitive channel $Na_V1.9$ has unique electrophysiological properties. $Na_V1.9$ currents were classified in nociceptive C-type sensory neurons in dorsal root ganglia, and in sensory neurons of the myenteric plexus.[15-18] In these cells, the channel opens at very low membrane potentials, mediates spontaneous excitation and shows slow activation and inactivation kinetics.[15,16,18,19] Thus, $Na_V1.9$ is thought to act as a regulator of subthreshold electrogenesis.[15,16,18-20]

These specific features of $Na_V1.9$ prompted us to investigate whether $Na_V1.9$ is involved in cell-autonomous, activity-dependent axon growth in motoneurons. When motoneurons are cultured from $Na_V1.9$ knockout mice, axons are much shorter than axons from wild-type mice (**Fig. 1B**).[6] This reduced axonal elongation in $Na_V1.9$-deficient motoneurons correlates with reduced numbers of spontaneous Ca^{2+} transients. Highest numbers of spontaneous calcium influx events are typically observed in growth cones and distal axons, but "global" as well as "local" calcium transients are strongly reduced in $Na_V1.9$-deficient motoneurons.[6]

Expression of $Na_V1.9$ in Young Motoneurons

In DRG neurons, $Na_V1.9$ expression levels are high[21] and $Na_V1.9$ specific currents are confirmed with the help of loss-of-function and gain-of-function experiments.[17] PCR data indicate that $Na_V1.9$ is widely expressed in excitable cells and neuroblastoma cell lines,[22,23] but, in contrast to data from DRG neurons or enteric sensory neurons, these expression data are not supported by a verification of voltage-dependent $Na_V1.9$ currents.

Earlier data showed no[21] or weak expression levels[22,23] for $Na_V1.9$ in the spinal cord. Therefore, we tested whether there are substantial differences in the expression levels of $Na_V1.9$ by comparing DRG neurons with the embryonic spinal cord, where young motoneurons reside, with motoneurons in culture and with in vivo motoneurons.

Quantitative PCR confirmed an exceptionally high expression rate for $Na_V1.9$ in DRG neurons[6] and revealed $Na_V1.9$ expression during early neuronal development. $Na_V1.9$ expression in DRG tissue starts already at embryonic day 12, increases to 1.150 $Na_V1.9$ transcripts per ng RNA at E18 and becomes very high in older mice (e.g., P19, 2.700 copies/ng RNA).[6] In spinal cord tissue, $Na_V1.9$ transcription gradually increases from E12 and peaks at late embryonic stages. At E18,

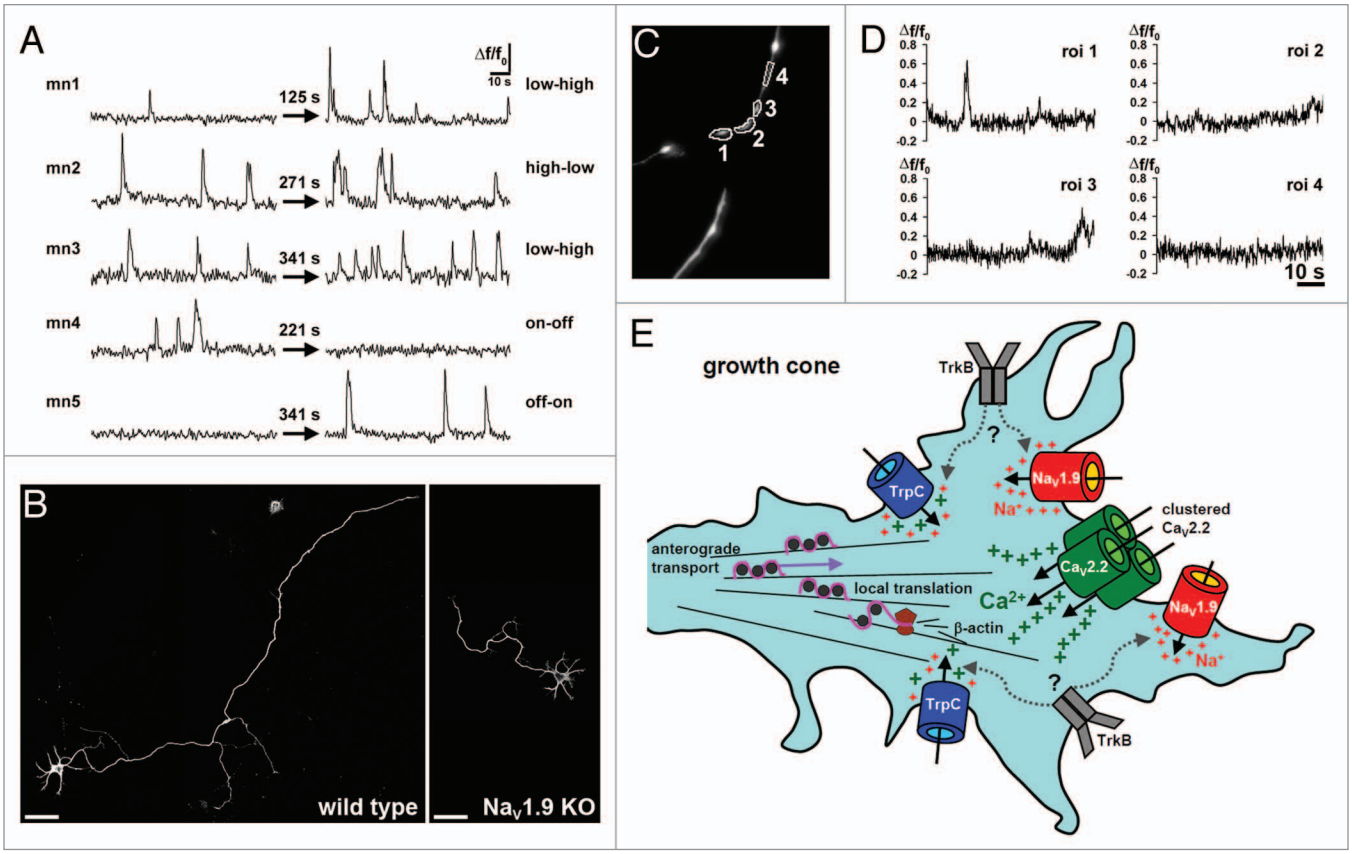

Figure 1. $Na_V1.9$ triggers spontaneous calcium influx in motoneurons and contributes to activity-depedent axon growth. (**A**) Characteristics of spontaneous Ca^{2+} transients in young cultured motoneurons from wild type mice. Motoneurons were cultivated for 5 d in vitro and afterwards loaded with the calcium indicator Oregon green BAPTA1. In live cell imaging experiments changes in fluorescence intensity ($\Delta f/f_0$) are illustrated from different cells. Calcium transients show variability in frequency and amplitude (mn1–3) and they change between on and off conformation within single cells (mn4 and 5). (**B**) Axon elongation is reduced in motoneurons from $Na_V1.9$ knockout mice. Representative motoneurons from wild-type and $Na_V1.9$ knockout mice are shown. Cells were cultured for 7 d in vitro. After anti-α-tubulin staining cells were analyzed by standard laser scanning microscopy. Bar: 40 μm. (**C**) Representative motoneuron at DIV5 is loaded with the calcium indicator Oregon green BAPTA1. (**D**) Calcium transients of roi1–roi4 as indicated in (**C**). Note here that local spontaneous calcium influx activity is observed in the growth cone only and does not spread to neighboring axonal regions. (**E**) Model for the interplay between local regulation of actin dynamics and ion channel gating in the plasma membrane in the growth cone of embryonic motoneurons. Actin mRNA is anterogradely transported from the soma into the axon and local synthesis of β-actin takes place in the growth cone. Axonal elongation is an activity-dependent process. The voltage-gated sodium channel $Na_V1.9$ opens spontaneously near the resting membrane potential and the influx of sodium leads to a depolarization cascade to gate clusters of the N-type voltage-gated calcium channels $Ca_V2.2$. The influx of free calcium supports axon growth by an unknown mechanism. The receptor tyrosine kinases TrkB might modulate the open probability of $Na_V1.9$ and/or leads to an opening of non-selective TrpC ion channels. Data were acquired during our previous study by Subramanian et al. (2012) with materials and methods described there in detail.[6]

$Na_V1.9$ transcription in the spinal cord is ~30-fold lower (37,3 copies/ng RNA) compared with DRG neurons.[6] $Na_V1.9$ transcripts are also found in young motoneurons in vivo. Laser-assisted microdissection of young motoneurons from the anterior horn of the spinal cord revealed a low abundance of $Na_V1.9$ transcripts, approximately 80x lower than in DRG neurons.[6]

This indicates that even low expression levels of $Na_V1.9$ are sufficient to augment neuronal excitability, to trigger spontaneous voltage-dependent calcium influx, and to support activity-dependent axon growth, at least in cultured embryonic motoneurons.

Early Sorting of $Na_V1.9$ Protein to Axons and Growth Cones

In young motoneurons, local $Na_V1.9$-dependent calcium signals are preferentially measured in axons and axonal growth cones. Therefore, $Na_V1.9$ protein should also be present in local regions within axons and growth cones. In order to identify the $Na_V1.9$ protein, an antibody was generated and tested on DRG tissue samples of the mouse. Western blots labeled endogenous mouse $Na_V1.9$ protein from DRG preparations and recombinant $Na_V1.9$ from different species at two bands of 180 kDa and approximately 280 kDa.[6] This typical western blot pattern is lost in preparations from a $Na_V1.9$ knock out mouse model. In this specific $Na_V1.9$ knock out mouse, introduced by Östman et al.,[17] the S4 voltage sensor segment of domain I in exon 4 and 5 of the $Na_V1.9$ gene was replaced by a neomycin resistance cassette, thus causing nonsense

mutations in exon 6. Immunofluorescence labeling of $Na_V1.9$ combined with super resolution imaging using stimulated emission depletion microscopy (STED) revealed that $Na_V1.9$ resides preferentially in axons and growth cones. Anti-$Na_V1.9$ immunolabels are distributed non-uniformly along the axon and are locally enriched at distinct axonal regions.[6] The spatial restriction of local calcium influx and the typical uneven distribution of $Na_V1.9$ protein predict mechanisms that help to localize $Na_V1.9$. Perhaps, the channel maintains this localization by an attachment to the cytoskeleton or by a diffusion barrier in axonal microdomains.

$Na_V1.9$ Might Act within Axonal Subcompartments

As discussed above, local translation of β-actin mRNA in growth cones is essential for proper growth cone function and is linked with activity-dependent axon growth.[5,7,12] The actin cytoskeleton in growth cones is essential for cell surface clustering of N-type calcium channels in distal axons, and activation of these channels is then triggered by $Na_V1.9$ activity (**Fig. 1E**).[5,6]

Local calcium transients in axonal regions are often observed within small, distinct axonal regions. **Figure 1C and D** shows an example of a "local" calcium transient, here imaged with the help of a high-affinity Ca^{2+} indicator at 10 Hz, in a single confocal plane. In this example, the calcium transient is only observed in a small torpedo-like growth cone of a cultured motoneuron and does not spread to adjacent axonal regions; hence it forms a microdomain or even a restricted axonal subcompartment. Cytoskeleton remodeling is upstream of increased local excitability in motoneurons. Rescue of β-actin dynamics in SMN-deficient motoneurons also rescues local calcium influx and activity-dependent axonal elongation. High motility at growth cones might be accompanied by a high elasticity of the lipid bilayer. The high motility of the lipid bilayer and the cytoskeleton in the "healthy" growth cone of motoneurons might increase the activation sensitivity of Na_Vs. For instance, $Na_V1.5$ is sensitive to mechanical stimuli and this causes shifts in activation and inactivation properties due to mechanical modulation of the voltage sensors.[24] Therefore, it is conceivable that proper cytoskeleton dynamics may directly influence the region-specific and local excitability of voltage-gated sodium channels in the growth cone.

Critical Aspects

There is a major critical aspect in our study. $Na_V1.9$ function was monitored indirectly and the electrophysiological properties of the channel have not been defined in motoneurons yet. Electrophysiological studies may help us to solve a critical issue regarding the use of pore blockers of voltage-gated sodium channels as growth inhibiting compounds. Axonal elongation of motoneurons is reduced in presence of 1–10 nM saxitoxin, more than 50 nM tetrodotoxin is necessary to get a comparable effect.[6] This indicates that STX has a higher affinity to $Na_V1.9$ than TTX. In line with this finding, it has already been described that low doses of STX reduce neurotrophin-induced neurite growth of young cultured hippocampal neurons of the rat.[25] In addition, reconstitution experiments mimicking neurotrophin-induced excitation in hippocampal neurons indicated that 10 nM STX inhibits neurotrophin-induced activation of $Na_V1.9$, while 50 nM TTX does not.[22,26]

Electrophysiological recordings with DRG neurons argue against the idea of a high specificity of saxitoxin to $Na_V1.9$. In DRGs, STX and TTX are both quite ineffective on persistent sodium currents attributed to the $Na_V1.9$ channel.[20] Dose-response experiments regarding voltage-dependent activation currents of $Na_V1.9$ revealed an inhibitor constant (K_i) value for TTX of 39 ± 9 μM.[16] Thus, it remains a puzzle why 10 nM STX has such a strong inhibitory effect on activity-dependent motoneuron axonal elongation[6] and neurite growth of hippocampal neurons.[22,25] When motoneurons are treated with 10 nM STX by fast perfusion with artificial cerebrospinal fluid (ACSF), local and global spontaneous calcium transients are rapidly blocked.[6] This effect is reversible. STX can be washed out by continuous perfusion with ACSF and spontaneous calcium influx recovers after 10–20 min.[6] STX and TTX seem to bind to all members of the Na_v1 family, and the differences in biological responses on neurite growth observed with these channel blockers at very low concentrations are not easy to explain. It may be that the growth-inhibiting effect of STX is observed, because it blocks sodium channels that are downstream of $Na_V1.9$ and have a high-affinity to STX. According to its properties, $Na_V1.2$[27] is a good candidate for being this sodium channel in young neurons, but for this we have no proof, yet.

In addition, it will be helpful to define the properties and constitution of these commercially available toxin preparations by independent methods. For this, a reconstitution system enabling the investigation of voltage-dependent and pharmacological properties of $Na_V1.9$ by heterologous expression, e.g., in HEK293 cells or another reconstitution cell line, would be helpful, but such an in vitro system is still a major issue in $Na_V1.9$ research.

Ion Channel Interplay in Growth Cones of Motoneurons

$Na_V1.8$ is the concomitant partner of $Na_V1.9$ in DRG neurons and seem not to be expressed in embryonic motoneurons[6] or healthy adult motor nerves.[28] We found 20-fold higher $Na_V1.5$ vs. $Na_V1.9$ expression levels in embryonic motoneurons.[6] $Na_V1.5$ has a quite low activation threshold[13] and would be an ideal physiological partner to potentiate $Na_V1.9$-mediated excitability. This led us to speculate that $Na_V1.9$ is first activating $Na_V1.5$, which then initiates a rapid depolarization and a fast voltage-dependent gating of $Ca_V2.2$ (**Fig. 1E**). During live imaging of spontaneous activity in motoneurons (minutes to hours), there are always phases of very low or very high activity and motoneurons switch between stages of no or low activity and stages of high activity (**Fig. 1A**). This raises the question how phases of high activity are stimulated. We have no evidence that increased levels of excitability in motoneurons are predictable or whether the channel opening is a stochastic event. In DRG neurons, $Na_V1.9$ action is regulated by intracellular signaling cascades.[15,17] In motoneurons, the tropomyosin receptor kinase B (TrkB)

might be a good candidate for regulating phases of higher vs. lower excitability. These receptors are expressed on cultured embryonic motoneurons and activation of these receptors by the neurotrophin brain-derived neurotrophic factor (BDNF) supports motoneuron survival.[29] In neurons of the hippocampus BDNF and TrkB are modulators of $Na_V1.9$ activity and are claimed to be involved in instructive effects of BDNF in synaptic plasticity.[22,26,30,31] To our opinion, it is unlikely that BDNF activates TrkB and induces $Na_V1.9$ activity and subsequent N-type channel gating in motoneurons. More likely is a model that includes signaling mechanisms such as transactivation of an intracellular domain of TrkB[32,33] and a subsequent increase of the open probability of $Na_V1.9$ (**Fig. 1E**).

It is also important to consider a potential function of transient receptor potential channels (Trp) in motoneuron growth cones. In young hippocampal neurons, TrpC5 channels are localized to neurite growth cones, are calcium permeable and regulate neurite extension and growth cone motility.[5] TrpC family members have also been identified in growth cones of cultured embryonic motoneurons.[5] It may be that kinase-active TrkB first acts via PLCγ recruitment to start a subsequent increase in excitability by a non-selective ion influx through TrpC channels. Such a mechanism was first observed in pontine neurons[34] and TrpC activity may offer a weak and local increase in excitability, $Na_V1.9$ activation and subsequent depolarization that gates voltage-dependent calcium influx.

In the last decade, investigating cell-autonomous growth mechanisms of motoneurons has offered important insights in the function of mammalian ion channels in activity-dependent growth. However, even though there is a concept on how ion channels in growth cones may interact, the molecular mechanism of the pivotal physiological role of increased free calcium in growth cones remains elusive.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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