Role of Na\textsubscript{v}1.9 in activity-dependent axon growth in motoneurons

Narayan Subramanian\textsuperscript{1,†,§}, Andrea Wetzel\textsuperscript{1,}, Benjamin Dombert\textsuperscript{1,}, Preeti Yadav\textsuperscript{1},
Steven Havlicek\textsuperscript{1,}, Sibylle Jablonka\textsuperscript{1,}, Mohammed A. Nassar\textsuperscript{2,§}, Robert Blum\textsuperscript{1,*,†} and Michael Sendtner\textsuperscript{1,*,†}

\textsuperscript{1}Institute for Clinical Neurobiology, University of Wuerzburg, Wuerzburg, Germany, and \textsuperscript{2}Molecular Nociception Group, Wolfson Institute for Biomedical Research, University College London, London WC1E 6BT, UK

Received April 20, 2012; Revised and Accepted May 18, 2012

Spontaneous neural activity promotes axon growth in many types of developing neurons, including motoneurons. In motoneurons from a mouse model of spinal muscular atrophy (SMA), defects in axonal growth and presynaptic function correlate with a reduced frequency of spontaneous Ca\textsuperscript{2+} transients in axons which are mediated by N-type Ca\textsuperscript{2+} channels. To characterize the mechanisms that initiate spontaneous Ca\textsuperscript{2+} transients, we investigated the role of voltage-gated sodium channels (VGSCs). We found that low concentrations of the VGSC inhibitors tetrodotoxin (TTX) and saxitoxin (STX) reduce the rate of axon growth in cultured embryonic mouse motoneurons without affecting their survival. STX was 5- to 10-fold more potent than TTX and Ca\textsuperscript{2+} imaging confirmed that low concentrations of STX strongly reduce the frequency of spontaneous Ca\textsuperscript{2+} transients in somatic and axonal regions. These findings suggest that the Na\textsubscript{v}1.9, a VGSC that opens at low thresholds, could act upstream of spontaneous Ca\textsuperscript{2+} transients. qPCR from cultured and laser-microdissected spinal cord motoneurons revealed abundant expression of Na\textsubscript{v}1.9. Na\textsubscript{v}1.9 protein is preferentially localized in axons and growth cones. Suppression of Na\textsubscript{v}1.9 expression reduced axon elongation. Motoneurons from Na\textsubscript{v}1.9\textsuperscript{−/−} mice showed the reduced axon growth in combination with reduced spontaneous Ca\textsuperscript{2+} transients in the soma and axon terminals. Thus, Na\textsubscript{v}1.9 function appears to be essential for activity-dependent axon growth, acting upstream of spontaneous Ca\textsuperscript{2+} elevation through voltage-gated calcium channels (VGCCs). Na\textsubscript{v}1.9 activation could therefore serve as a target for modulating axonal regeneration in motoneuron diseases such as SMA in which presynaptic activity of VGCCs is reduced.

INTRODUCTION

Axons of developing motoneurons grow long distances before they make synaptic contacts with their target tissue, the skeletal muscle (1). During this period, motoneurons depend on neurotrophic factors for their survival (2–4). Cultured embryonic motoneurons also need neurotrophic factors for survival and neurite growth, thus allowing the analysis of signaling pathways for axon elongation and differentiation of presynaptic structures within axonal growth cones (1,5–7). Embryonic motoneurons exhibit spontaneous activity at early stages, before they make synaptic contact with skeletal muscles (8–12). Spontaneous Ca\textsuperscript{2+} elevation is an evolutionary conserved phenomenon in growth and differentiation of neurons (10,11,13–16) and is also observed in cultured mouse motoneurons (17). In motoneurons, spontaneous Ca\textsuperscript{2+} transients in axons and axonal growth cones contribute to axon extension and presynaptic differentiation (17). These characteristics are
also pathophysiologically relevant. Spontaneous Ca\(^{2+}\) transients are reduced in motoneurons from a mouse model of spinal muscular atrophy (SMA) (17), the predominant form of motoneuron disease in children and young adults. The reduced frequency of Ca\(^{2+}\) transients correlates with a reduced axon elongation and defective presynaptic differentiation \textit{in vivo} (18–20).

Survival and axon elongation of cultured motoneurons are strongly influenced by the availability of extracellular matrix proteins (1,21). Laminin-111 supports axon elongation in cultured motoneurons, while laminin-211/221 preparations reduce axon elongation (17). The synapse-specific \(\beta\)-2-chain in laminin-221 mediates differentiation of presynaptic active zones by the direct interaction with Ca\(_v\)2.2, an N-type voltage-gated calcium channel (VGCC) (22). In \(Smn^{−/−}\)SMN2 mice, defects in the clustering of the Ca\(_v\)2.2 are observed in axon terminals (17).

We investigated the role of voltage-gated sodium channels (VGSCs) in activity-dependent axon elongation in cultured motoneurons and found that TTX and STX, specific pore-blockers of VGSCs, reduce spontaneous Ca\(^{2+}\) transients and also axon growth. High sensitivity to STX indicated that Na\(_v\)1.9 might contribute to spontaneous excitability in embryonic motoneurons. Indeed, motoneurons from Na\(_v\)1.9 knockout mice have shorter axons, and exhibit a reduced frequency of spontaneous Ca\(^{2+}\) transients. Taken together, these data indicate that Na\(_v\)1.9 plays a central role for spontaneous excitability that regulates the axonal growth in developing motoneurons.

**RESULTS**

**VGSCs modulate axon growth in cultured motoneurons**

To investigate whether VGSCs are involved in activity-dependent axon elongation, we tested the effects of the sodium-channel inhibitors tetrodotoxin (TTX) and saxitoxin (STX) in cultures of isolated spinal motoneurons. TTX binds all channels of the Na\(_v\)1-family with high affinity, except the TTX-insensitive channels (23). The TTX-insensitive channels Na\(_v\)1.8 and Na\(_v\)1.9 carry a serine (S) residue, whereas Na\(_v\)1.5 carries a cysteine residue in a critical TTX affinity motif, which is close to the selectivity determining (inner ring residues Asp, Glu, Lys, and Ala) motif of VGSCs (23–25). All other Na\(_v\)1-family members carry a phenylalanine (F) or tyrosine (Y) residue at the homologous site of domain DI, SS2-segment of the \(\alpha\)-subunit (23,24). The determination of the equilibrium binding free energy of TTX and STX in dependence of these critical residues suggested that non-aromatic residues at the outer pore binding site of the channel shift the affinity of TTX-binding to lower values, while STX affinity is less affected (25).

Motoneurons were isolated from lumbar spinal cord of E14 C57/BL6 mice and plated at low density on laminin-111. These cell culture conditions allow minimal cell–cell contact and maximal axon extension of the cultured motoneurons (26). At DIV 7, motoneurons were stained with \(\alpha\)-Tau antibody (Fig. 1A and B) and the axon length was measured (Fig. 1C and D). Motoneurons treated with 1–10\(\text{\textmu}\)M STX showed a significant decrease in the axon length (Fig. 1A and C). In contrast, TTX was not effective at the same low concentrations. The reduced axon length was observed only at TTX concentration of 50\(\text{\textmu}\)M (Fig. 1B and D) and higher (data not shown). Neither STX nor TTX had an influence on motoneuron survival at the same concentrations that led to the reduced axon growth (Fig. 1E and F). The number and length of motoneuron dendrites (Fig. 1G) were not affected when motoneurons were treated with 10\(\text{\textmu}\)M TTX or 10\(\text{\textmu}\)M STX (Fig. 1H and I).

Figure 1. The axon elongation is reduced by pore blockers of VGSCs. (A and B) Axon length analysis of cultured motoneurons after anti-Tau staining. Analyzed cells were grown in the presence of saxitoxin (STX) and tetrodotoxin (TTX). (C and D) The axon length of motoneurons at indicated conditions. STX: 1 nM (\(n = 168\)), 5 nM (\(n = 190\)), 10 nM (\(n = 183\)) and TTX: 5 nM (\(n = 158\)), 10 nM (\(n = 110\)), 50 nM (\(n = 140\)). Control\(\_\)STX treatments: \(n = 183\), control\(\_\)TTX treatments: \(n = 183\), control\(\_\)TTX treatments: \(n = 101\). Bar (A–D): 150\(\mu\)m. (E and F) Motoneuron survival in the presence or absence of neurotrophic factors, at indicated concentrations of STX and TTX (\(n = 3\) independent cultures). (G) Dendrite analysis after anti-Tau staining. Bar: 15\(\mu\)m. The dendrite length (H) and number of dendrites (I) of cultured motoneurons treated with or without STX (10 nM) and TTX (10 nM) values. Dendritic length: control, \(n = 1182\); 10 nM TTX, \(n = 976\); 10 nM STX, \(n = 501\); number of dendrites, control, \(n = 230\); 10 nM TTX, \(n = 205\); 10 nM STX, \(n = 81\). Results represent the mean \pm SEM of pooled data from three independent experiments, \(n\), number of motoneurons that were scored in total from control or toxin treated motoneurons. **\(P < 0.0001\); **\(P < 0.01\) tested by one-way ANOVA, Bonferroni post hoc test.
VGSC trigger spontaneous Ca\textsuperscript{2+} transients in cultured motoneurons

In cultured motoneurons, the axon growth is slow in a first phase until DIV 3, reaching about 100–150 μm of length. After DIV 3, axons grow fast, and they reach an average length of 600–800 μm at DIV 7. The frequency of local Ca\textsuperscript{2+} transients in axonal growth cones correlates with the speed of axon elongation during these different time periods in culture (17). To analyze the role of VGSCs in spontaneous excitability of motoneurons, we measured spontaneous Ca\textsuperscript{2+} transients with the calcium indicator dye Oregon-green BAPTA-1-AM (K\textsubscript{d}: 130 nm) between DIV 3 and DIV 4, when the speed of axon elongation is highest. Initial experiments at DIV 3 showed that motoneurons were heterogeneous with respect to spontaneous activity, some showing low activity with rare Ca\textsuperscript{2+} transients and others with four or even more spontaneous transients per minute (not shown). Motoneurons can switch between such activity states (not shown). As suggested previously (15,27), we term broad spontaneous activity that results in synchronous transients in all parts of the cell as ‘global activity’, while locally restricted Ca\textsuperscript{2+} transients in all parts of the cell are termed ‘local activity’. An example for such global activity is presented in Figure 2A and Supplementary Material, Movie S1, showing synchronous transients in three regions of interest (roi), namely the axon initiation segment (roi1), the axon (roi2) and the axonal growth cone (roi3). Figure 2B and Supplementary Material, movie S2 show an example for a motoneuron exhibiting spontaneous, local Ca\textsuperscript{2+} transients in the axon (roi2–4). The somatodendritic region was silent (roi1) when these Ca\textsuperscript{2+} transients were measured in the same cell.

From previous studies (17) we know that spontaneous Ca\textsuperscript{2+} influx to motoneurons is blocked by Ω-conotoxin. In accordance with this, 30 nM Ω-conotoxin MVIIa fully blocked spontaneous Ca\textsuperscript{2+} transients (Fig. 2C1 and C2). Ω-Conotoxin MVIIa has a high affinity to N-type VGCCs, while much higher concentrations lead to a block of other VGCCs (28). We then tested the sodium-channel pore blockers TTX and STX to figure out whether the opening of VGSCs triggers Ca\textsuperscript{2+} influx through VGCCs in motoneurons (Fig. 2D and E, Fig. 3). Low concentrations of STX (10 nM) had a strong inhibitory function on spontaneous Ca\textsuperscript{2+} influx (Figs 2D and 3B). TTX was less effective at 10 nM (Figs 2E and 3C), but showed a strong block of spontaneous activity when used at 100 nM (Fig. 3A and D). For quantitative analysis of VGSC function in triggering spontaneous Ca\textsuperscript{2+} transients, we analyzed motoneurons in the active state and monitored Ca\textsuperscript{2+} transients during a period of 40–60 min (long-term example is shown in Fig. 3A). Then, we analyzed transients in intervals (i) before the sodium-channel blocker treatment (Fig. 3A; spontaneous), (ii) in the presence of either STX or TTX, (iii) during the wash out of the channel blockers and (iv) 10 min after STX or TTX treatment, when activity recovers (spontaneous recovery) from toxin treatment (Fig. 3A). Spontaneous Ca\textsuperscript{2+} transients (Fig. 3B) were blocked by 10 nM STX in all cellular regions (soma, distal axon and growth cone), whereas TTX was less efficient to block these transients at the same concentration (Fig. 3C), in particular in the axon and axonal growth cone. The concentration of TTX necessary for an almost complete blockage of spontaneous Ca\textsuperscript{2+} transients was 100 nM (Fig. 3A and D). The effect was reversible when the inhibitors were washed out by a 10-fold artificial cerebrospinal fluid (ACSF) exchange (volume/min) over a period of 10 min. The experiments show that sodium-channel inhibitors reduce global and local Ca\textsuperscript{2+} transients in all cellular regions of motoneurons. Thus, the observation that low concentrations of STX inhibit axon elongation (Fig. 1) correlates with a reduced rate of Ca\textsuperscript{2+} transients under acute STX application.

Na\textsubscript{v}1.9 is expressed in cultured motoneurons

To identify the VGSCs that are responsible for triggering Ca\textsuperscript{2+} transients in developing motoneurons, we concentrated on Na\textsubscript{v}1.9. Several lines of evidence point to Na\textsubscript{v}1.9 as the...
TTX-resistant VGSCs Na\textsubscript{V}1.5, Na\textsubscript{V}1.8, and Na\textsubscript{V}1.9 by neuron cultures, substantial expression of Na\textsubscript{V}1.9 (Fig. 4B, external standard curves (10 ng–500 pg heart RNA). Na\textsubscript{V}1.9 RNA, 2 ng–100 pg, served as reference. NaV1.5 is prominent expression increased continuously between E14 and E18 (E18; spinal cord: 163 copies/10 ng RNA; DRG: 11 580 copies/10 ng RNA; Table1; 0.11% of the amount of GAPDH and Na\textsubscript{V}1.5 (left panel) are expressed in cultured motoneurons. Na\textsubscript{V}1.8 was not detected (left panel). (C and D) Real-time monitoring of the fluorescence emission of Sybr Green1 during PCR amplification of Na\textsubscript{V}1.5 (C) and Na\textsubscript{V}1.9 (D). (E and F) Na\textsubscript{V}1.9 expression in motoneurons and DRG neurons in situ. Real-time amplification curves of Na\textsubscript{V}1.9 cDNA from laser-dissected motoneurons of the spinal cord (E) or DRG neurons (G) and corresponding Na\textsubscript{V}1.9-specific amplification products (F and H). In Na\textsubscript{V}1.9 amplification, serial dilutions of spinal cord RNA served as external control (black lines in D, E, G); for Na\textsubscript{V}1.5, heart RNA was used as control (black lines, C).

most likely candidate for initiating VGSC-dependent spontaneous Ca\textsuperscript{2+} fluxes through VGCCs. Na\textsubscript{V}1.9 has a low activation threshold and is able to mediate spontaneous excitation at resting potential levels, as shown previously in dorsal root ganglia (DRGs) and myenteric sensory neurons (29–32).

We therefore amplified transcripts encoding the TTX-resistant VGSCs Na\textsubscript{V}1.5, Na\textsubscript{V}1.8, and Na\textsubscript{V}1.9 by efficiency-controlled quantitative RT–PCR (qRT–PCR) and determined the number of Na\textsubscript{V}1.9 transcripts in the developing spinal cord, in DRGs and cultured motoneurons (Fig. 4A–D). In Fig. 4, representative amplification products and real-time PCR amplification curves are shown. For Na\textsubscript{V}1.9 amplification, external standard dilution curves with E18 spinal cord RNA, 2 ng – 100 pg, served as reference. Na\textsubscript{V}1.5 is prominent for its function in triggering action potentials in the heart (24). Therefore, the heart RNA was used to produce Na\textsubscript{V}1.5-specific external standard curves (10 ng – 500 pg heart RNA). Na\textsubscript{V}1.9 transcripts were detectable at DIV 3, before toxin treatment (ctr), in the presence or absence of 10 nM STX (n = 12, C), 10 nM TTX (n = 11, D) and 10 min after ACSF perfusion wash, during the phase of recovery of spontaneous activity. Ca\textsuperscript{2+} transients were counted in the somatodendritic area, the distal axon and in growth cones. The number of local spontaneous Ca\textsuperscript{2+} transients is higher in growth cones compared with somatodendritic areas. 10 nM STX and 100 nM TTX block spontaneous activity in all cellular regions. Results represent the mean ± SEM of pooled data. Cultures\textsubscript{STX} = 5, cultures\textsubscript{TTX} = 4, *P < 0.0001, **P < 0.01, *P < 0.05 tested by one-way ANOVA-nonparametric Kruskal–Wallis test, Dunn’s post test.

To reveal expression of Na\textsubscript{V}1.9 in spinal motoneurons in situ, we microdissected motoneurons from spinal cord by laser capture microscopy at postnatal day 2 (P2), a time point that corresponds to the age of motoneurons that had been isolated at E14 and maintained in culture for 7 days.
NaV1.9 (71n) ImmR showed a distinct overlap with ImmR
dogenous mNaV1.9 also showed an
To detect NaV1.9 protein in cultured motoneurons, anti-mouse
protein in tissue
(31). In western blots from
frameshift mutation introduces stop codons within exon 6
the human Na V1.9 protein was preferentially
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NaV1.9

copies of NaV1.9 versus 100 copies of GAPDH. Expression
in motoneurons then decreases until P4 (NaV1.9
versus GAPDH; 0.13%; Fig. 4E). At that stage, the relative expression
of NaV1.9 molecules versus GAPDH; 0.13%; Fig. 4E).

NaV1.9 depletion reduces axon elongation
To test the role of NaV1.9 for axon elongation, lentiviral
shRNA expression vectors were designed and used to suppress
the expression of this channel in motoneurons. The fluorescent
protein Tandem-tomato (TDTomato) was co-expressed as infection
control (Fig. 7). As a control for the efficacy of repression,
shRNA-mediated NaV1.9 knockdown was verified by western blot analysis of HEK293T cells expressing mouse
NaV1.9 (Fig. 7A; sh 63; sh 3028). NaV1.9 expression
was not reduced in cells expressing empty lentiviral vectors or constructs expressing missense control shRNA (mis 63) in which four bases were exchanged (Fig. 7A; sh vector, mis 63). Then, lentiviral vectors were used to infect motoneurons at DIV 1. Infected motoneurons were identified at DIV 7 and the axon length was analyzed. In sh 63-RNA-treated motoneurons, the axon length was reduced by 43% in comparison to uninfected motoneurons in the same cultures or control cultures treated with missense shRNA-expressing virus (Fig. 7B, red). A second set of lentivirally applied shRNA against the 3’-untranslated region of NaV1.9, with GFP as infection control, showed 46% reduction in axon elongation in motoneurons at DIV 7 (Fig. 7B, green).

Motoneurons from NaV1.9<sup>−/−</sup> mice exhibit shorter axons and reduced frequency of Ca<sup>2+</sup> transients
We also isolated motoneurons from NaV1.9<sup>−/−</sup> knockout mice (31) and investigated them in comparison to strain-matched wild-type controls. No anti-NaV1.9 ImmR was observed in motoneurons at DIV 5 (Fig. 8A). NaV1.9<sup>−/−</sup> motoneurons

Anti-NaV1.9 antibody and identification of mouse NaV1.9 protein in tissue
To detect NaV1.9 protein in cultured motoneurons, anti-mouse
NaV1.9 antibodies were raised in rabbits. The C-terminal end
of mouse NaV1.9 was used for immunization (Fig. 5A). For control experiments, full-length mouse NaV1.9 was cloned
into a modified pcDNA3 vector and served as control. The rabbit antiserum [NaV1.9 (71n)] that was raised against
Anti-NaV1.9 antibody and identification of mouse NaV1.9 proteins
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Table 1. Absolute quantification of NaV1.9 transcripts

| Origin of RNA | Spinal cord | DRG | P1 | P19 | MN | DIV7 |
|---------------|-------------|-----|----|-----|----|------|
| Developmental state | E12 | E14 | E15 | E16 | E18 | P1 | E12 | E14 | E15 | E16 | E18 | P19 |
| Copies NaV1.9/10 ng RNA | 2 | 8 | 48 | 92 | 373 | 207 | 18 | 70 | 3499 | 4965 | 11580 | 27015 | 26 |
| Copies NaV1.9/copy GAPDH × 100 | 0.007 | 0.02 | 0.2 | 0.18 | 0.7 | 0.43 | 0.04 | 0.12 | 11.79 | 15.39 | 56.03 | 56.03 | 12.8 | 0.11 |

until they were analyzed. In addition, motoneurons were analyzed at P4. DRG neurons served as reference and GAPDH as denominator in all samples. As shown in Figure 4E and F, the spinal cord motoneurons express NaV1.9 transcript at P2. In P2 motoneurons in situ, the number of NaV1.9 transcripts is 0.44% of GAPDH transcripts, while in DRG neurons, 35 copies of NaV1.9 correspond to 100 copies of GAPDH. Expression in motoneurons then decreases until P4 (NaV1.9 versus GAPDH: 0.13%; Fig. 4E). At that stage, the relative expression of NaV1.9 molecules versus GAPDH; 0.13%; Fig. 4E).

NaV1.9 protein is localized in axons and axonal growth cones
To localize NaV1.9 in cultured motoneurons, we used stimulated emission depletion (STED) microscopy and combined
this technique with standard confocal laser scanning microscopy for the detection of F-Actin and α-Tubulin as structural markers. NaV1.9 protein was not homogenously distributed in axons. When cultured motoneurons were stained with anti-NaV1.9, ImmR was detectable along the axon but the staining appeared to be enriched at some sites (arrows in Fig. 6A and B). In axons and growth cones, NaV1.9 ImmR was high in the distal axons and also found in small protrusions (right arrow in Fig. 6C). Single confocal STED planes revealed that small punctate anti-NaV1.9 ImmR was lining along the cell surface of growth cones (arrows in Fig. 6D, upper and lower panel).

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shown highly reduced rates of spontaneous Ca\(^{2+}\) transients at DIV 3, in the soma (−75%), distal axonal regions (−82%) and growth cones (−83%) (Fig. 8B), indicating that the loss of Na\(_{V}1.9\) affects global and local Ca\(^{2+}\) transients of spontaneous excitability. Dendritic elongation (Fig. 8C) and motoneuron survival (Fig. 8D) were unaffected. However, axon elongation of Na\(_{V}1.9\)−/− motoneurons was reduced by 38% compared with wild-type littermates (Fig. 8E).

We then investigated whether the reduction of spontaneous Ca\(^{2+}\) transients observed in motoneurons from a mouse model of SMA (Smn\(^{-/-}\)/SMN2) (17) is also influenced by inhibition of Na\(_{V}1.9\) and other VGSCs. In Smn\(^{-/-}\)/SMN2 mice, spontaneous Ca\(^{2+}\) transients are reduced because of defects in the clustering of the CaV2.2 in axon terminals thus leading to a reduced axonal elongation (17). When Smn\(^{-/-}\)/SMN2 motoneurons were treated with 10 nM STX, axonal elongation remained unchanged, showing that function-blocking concentrations of STX have no additional growth inhibiting effect on Smn\(^{-/-}\)/SMN2 motoneurons (Fig. 8F). This experiment raised the question whether Na\(_{V}1.9\) expression and protein distribution has changed in Smn\(^{-/-}\)/SMN2 motoneurons. Immunolocalization of Na\(_{V}1.9\) in Smn\(^{-/-}\)/SMN2 motoneurons revealed Na\(_{V}1.9\) concentration in axons and axon terminals (Fig. 9A, four representative distal axons and growth cones are shown).

Next, we performed qPCR on RNA samples harvested from four independent single-embryo motoneuron cultures of Smn\(^{-/-}\)/SMN2 motoneurons. Expression levels of Na\(_{V}1.9\) at DIV 7 in Smn\(^{-/-}\)/SMN2 motoneurons are comparable in control and SMN-deficient motoneurons (Fig. 9B and C).

**DISCUSSION**

Spontaneous activity plays an important role during development of the nervous system when neurons make connections and synaptic networks are shaped (10,11,13–16). During development, motoneurons become spontaneously active long before they make synaptic contacts with skeletal muscle (9,16,34). In culture, embryonic mouse motoneurons display spontaneous Ca\(^{2+}\) transients that are important for axon elongation and growth cone differentiation (17). Downstream Ca\(^{2+}\)-dependent signaling pathways modulate microfilament and microtubule networks that mediate the effects of spontaneous excitability on the axon growth (35). Blockade of VGCC by Ω-conotoxin leads to ~40% reduced axon elongation in cultured embryonic mouse motoneurons on laminin-111 (17), indicating that depolarization-induced Ca\(^{2+}\) influx plays a major role in axon elongation.

Here, we provide evidence that the VGSC Na\(_{V}1.9\) acts as an upstream trigger of spontaneous excitatory Ca\(^{2+}\) influx in motoneurons and thus modulates the rate of axon elongation, without affecting motoneuron survival. Apparently, other members of the Na\(_{V}\) family cannot compensate for the deficiency of Na\(_{V}1.9\). This is not unexpected. Among the nine members of the Na\(_{V}\) family, Na\(_{V}1.9\) exhibits a specific property in that it opens spontaneously when neurons are kept close to the resting potential (29–32). The effect on axon elongation

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**Figure 5.** Anti-mouse Na\(_{V}1.9\) antibody. (A) A peptide was deduced from the carboxyterminal end of mouse Na\(_{V}1.9\) (red) and used for immunization of rabbits. The selected peptide sequence is specific for Na\(_{V}1.9\). Differences to rat or human Na\(_{V}1.9\) are marked (yellow). (B) Anti-Na\(_{V}1.9\) (71n) recognizes recombinant human Na\(_{V}1.9\) and mouse Na\(_{V}1.9\) in western blot analysis. As vector control, GFP-expressing vectors were used. (C) Immunoreactivity of anti-Na\(_{V}1.9\) (71n) on recombinant Na\(_{V}1.9\) is blocked by the corresponding 71n immunization peptide. Bar: 100 µm. (D) Stable expression of mNa\(_{V}1.9\) in HEK293T cells reveals a pronounced overlap of anti-Na\(_{V}1.9\) (71n) and anti-pan Na\(_{V}1.9\) immunoreactivity. Bar: 15 µm. (E) Endogenous mouse Na\(_{V}1.9\) from lumbar and thoracic DRGs is displayed by two bands at M\(_{w}\) 180 kDa and ~280 kDa. (F) Endogenous mouse Na\(_{V}1.9\) in wild-type DRGs and recombinant Na\(_{V}1.9\) in the stable cell line 293-mNa\(_{V}1.9\) are displayed by the typical double-band pattern bands at M\(_{w}\) 180 kDa and ~280 kDa. Both bands are lost in Na\(_{V}1.9\)−/− mice (representative for n = 4). Anti-Trk (tropomyosin-related kinase) antibodies served as loading control for DRG tissue.
observed in motoneurons from NaV1.9 knockout mice and in motoneurons in which Na V1.9 is acutely suppressed by shRNA was similar. This argues against the possibility that deficiency of NaV1.9 in knockout mice leads to a developmental defect that indirectly impairs spontaneous activity. Furthermore, the blockage of voltage-gated Na\(^{+}\) channels by STX and, at least at higher concentrations, that by TTX provokes a similar extent in the reduction of spontaneous activity and axon elongation. This appears remarkable as TTX and STX should act on a broad spectrum of VGSCs, and the reduction in spontaneous activity and axon elongation observed with these pharmacological inhibitors is not higher than after NaV1.9 knockout or shRNA-mediated knockdown. This indicates that NaV1.9 is the predominant VGSC that triggers spontaneous activity for axon elongation. Spontaneous Ca\(^{2+}\) transients in embryonic motoneurons are either locally restricted or globally distributed over the whole cell. In NaV1.9\(^{-/-}\) motoneurons, Ca\(^{2+}\) transients are massively reduced at DIV 3–4, both global transients that are synchronously observed in all parts of the cell and local transients that are seen only in axons.

Voltage-gated Ca\(^{2+}\) influx modulates the axon growth in motoneurons that are cultured at low-density so that the cells cannot make synaptic contact with each other. Furthermore, during the monitoring of Ca\(^{2+}\) influx, cells were kept under fast perfusion in an ACSF extracellular solution with 3 mM potassium. These characteristics would hardly allow motoneuron depolarization with ligand-dependent stimuli by synaptic input or excitatory neurotransmitters in the medium.

NaV1.9 is responsible for prolonged sodium influx into a variety of neurons (29,31,33,36,37) and thus can depolarize the cells so that Ca\(^{2+}\) influx through VGCCs can occur with high efficacy. Moreover, Na V1.9 is predominantly localized in axons and axonal growth cones so that opening of VGCCs by NaV1.9 is facilitated even under conditions when opening of Na V1.9 provokes only a local depolarization in axonal growth cones. In embryonic chick motoneurons, a persistent sodium current is involved in the generation of spontaneous excitatory bursts (13) and experiments by Kastenenka and Landmesser have demonstrated that spinal motor circuits are sensitive to the precise frequency and pattern of spontaneous activity (38). The molecular identity of the persistent sodium current which is involved in normal firing patterns of chick motoneurons has not been identified and it is tempting to speculate that NaV1.9 plays a central role. As chick NaV1.9 (NM_001192868) is 79% identical with mouse NaV1.9 (NM_011887), it may be possible that NaV1.9 is the upstream trigger of spontaneous Ca\(^{2+}\) bursts during motoneuron path-finding in the chick.

A critical issue is how the opening of NaV1.9 is regulated. Studies on fast (milliseconds) excitatory transients after brain-derived neurotrophic factor (BDNF) application (39)
suggested that Na\textsubscript{v}1.9 can be opened in central neurons by BDNF via TrkB activation (33,40). However, the existence of such BDNF–induced fast Na\textsuperscript{+} currents has become controversial (41). In particular, it has been noted that the kinetics of BDNF–induced fast Na\textsuperscript{+} currents are Fast activation Na\textsubscript{v}1.9 (42). The motoneurons used in our study were continuously cultured with BDNF to support their survival. Thus, it is possible that TrkB signaling is involved in the cascades that lead to opening of VGCCs through Na\textsubscript{v}1.9. It is unlikely that Ca\textsuperscript{2+} elevation in motoneurons is caused by spontaneously released BDNF from cells in these cultures, given that BDNF is present at concentrations of 10 ng/ml that saturate TrkB receptors on the cell surface. However, it is possible that other signaling mechanisms such as transactivation of TrkB receptors on the cell surface. Therefore, it is possible that other signaling mechanisms such as transactivation of TrkB receptors on the cell surface contribute to the opening probability of Na\textsubscript{v}1.9 (31,45) and therefore might increase the local, growth-mediating excitability of motoneurons. It is unresolved whether Na\textsubscript{v}1.9 is opened by direct interaction with TrkB or indirectly via other signaling pathways or TrkB-dependent rapid changes in ion fluxes that alter the resting potential in such a way that Na\textsubscript{v}1.9 can open.

Figure 7. Na\textsubscript{v}1.9 regulates axon elongation of cultured motoneurons. (A) Knockdown of recombinant mouse Na\textsubscript{v}1.9 by shRNA (sh 63, sh 3028), but not miRna shRNA (miR 63) or empty sh-expression vector (sh vector). Western blot analysis after transient transfection in HEK293T cells using anti-Na\textsubscript{v}1.9 (71n). Loading control: γ-Adaptin (γAd). (B) Axon length analysis after lentiviral shRNA expression. Red bars: lentiviral construct with cytomegalovirus (CMV)-TDtomato-H1-shRNA: uninfected (n = 67), mis 63 Na\textsubscript{v}1.9 (n = 108), sh 63 Na\textsubscript{v}1.9 (n = 105). Green bars: U6-shRNA-CMV-eGFP: uninfected (n = 134), mis 3' UTR Na\textsubscript{v}1.9 (n = 65), sh 3' UTR Na\textsubscript{v}1.9 (n = 65). Results represent the mean ± SEM of pooled data from three independent experiments, n, number of motoneurons. Figure 8. Reduced axon growth and less spontaneous Ca\textsuperscript{2+} transients in motoneurons from Na\textsubscript{v}1.9\textsuperscript{+/−} mice. (A) Na\textsubscript{v}1.9 (71n) antiserum reveals lack of Na\textsubscript{v}1.9 protein (arrows upper panel) in motoneurons (DIV 5) of Na\textsubscript{v}1.9\textsuperscript{+/−} mice (lower panel). (B) Spontaneous Ca\textsuperscript{2+} transients are reduced in the soma, distal axon and growth cone of motoneurons cultured from Na\textsubscript{v}1.9\textsuperscript{+/−} mice (+/−; n = 80; Na\textsubscript{v}1.9\textsuperscript{−/−}; n = 138; four independent cultures). (C) The dendrite length is unaffected in Na\textsubscript{v}1.9\textsuperscript{−/−} motoneurons (+/−: n = 752 dendrites; Na\textsubscript{v}1.9\textsuperscript{−/−}: n = 1049 dendrites; three independent cultures). (D) Motoneuron survival in the presence of the neurotrophic factors BDNF and ciliary neurotrophic factor (B and C) is not disturbed in Na\textsubscript{v}1.9\textsuperscript{+/−} motoneurons (n = 2 independent cultures). (E) The axon elongation is reduced in Na\textsubscript{v}1.9\textsuperscript{−/−} motoneurons (+/−: n = 286; Na\textsubscript{v}1.9\textsuperscript{−/−}: n = 447; 3 independent cultures). (F) Axonal elongation of motoneurons of Smn (−/−)/SMN2 mice is not reduced by 10 nM STX treatment (ctr: n = 197, mean length: 433 μm; 10 nM STX: n = 241, mean length: 439 μm; n = 3 coverslips). Figure 7.
The apparent effect of Na\textsubscript{v}1.9 on axon elongation in cultured embryonic motoneurons appears to be not reflected by the relatively mild phenotype of Na\textsubscript{v}1.9 knockout mice (31,46,47). These mice develop normally, and show only defects in pain perception, but no apparent functional defect that is caused by a reduced axon growth of motoneurons to reach their target, the skeletal muscles (46). This is not surprising when the effects of Na\textsubscript{v}1.9 knockout are compared with models for SMA in which Smn expression is reduced. Isolated motoneurons from a mouse model of SMA (48) show severe defects in Ca\textsuperscript{2+} transients in axons and axonal growth cones, and the reduced frequency of Ca\textsuperscript{2+} transients also correlates with a reduced axon elongation (17). However, the axons of the Smn-deficient motoneurons reach skeletal muscle, and no reduction in neuromuscular endplate formation is observed in mouse embryos with Smn deficiency (49,50). However, when Smn (51) or Smn-interacting proteins such as hnRNPR (52) are knocked down by Morpholino technologies in zebrafish, the axons of motoneurons show severe defects in axon elongation and pathfinding. This indicates that animal models in which the motor axon growth occurs within a short time window suffer more from these genetic defects than developing mice in which axon elongation, pathfinding and synapse formation occur over a relatively prolonged time period. Thus, a failsafe mechanism seems to exist that compensates for the reduced speed of axon elongation and allows motoneurons to reach their target.

The signaling cascades leading to motoneuron differentiation and motoneuron axon elongation are also of central interest for the development of therapies for motoneuron diseases, in particular SMA in which a defect in synapse maintenance appears as a central pathophysiological mechanism (17,48–50,53). Previous studies indicate that intracellular signaling pathways can activate persistent sodium currents by Na\textsubscript{v}1.9 (31,45). This raises the hope that pharmacological activation to increase the opening probability of Na\textsubscript{v}1.9 could be a way to stimulate axon regeneration and maintenance.

**MATERIALS AND METHODS**

**Animals**

All experimental procedures were done in accordance with European Union guidelines, as approved by our institutional...
animal care and utilization committee. The following mouse lines were used in this study: C57BL/6J mice; *Naᵥ1.9*−/− mice on a C57BL/6J background (31) and *Smn*−/−/SMN2 mice (48) on a FVB/Ncrl background.

**Cell culture**

Spinal motoneurons were isolated from 14-day-old C57/BL6/J mouse embryos of either sex, enriched by panning using an antibody against the p75<sup>NTR</sup> receptor and plated at a density of 1500 cells/well on laminin-coated coverslips as described previously (26). Cells were grown in neurobasal medium containing B27 supplement, 10% heat inactivated horse serum, 500 µM Glutamax (Invitrogen), 10 ng/ml BDNF and 10 ng/ml ciliary neurotropic factor. Medium was replaced after 24 h and then every second day. ß-Conotoxins MVIIa (Sigma), tetrodotoxin (Sigma, Ascent Scientific) and saxitoxin (Sigma) was added at indicated concentrations. Motoneuron survival was determined after 7 days in culture as described earlier (54). Motoneurons from *Naᵥ1.9* or *Smn*/*SMN2* mouse models were prepared from single embryos. Genotyping was then performed from corresponding tissue samples.

**Quantitative real-time reverse transcriptase–polymerase chain reaction**

Reverse transcription, primer selection and qPCR were performed with minor modifications as described in earlier studies (55–57). RNA from mouse embryonic DRG and the spinal cord was isolated by standard protocols using RNeasy Plus-Mini-Kit (Qiagen), with the help of a silent cruscher (Heidolph). RNA from single-embryo motoneuron cultures (*Smn*/*SMN2* RNA samples) was prepared with the RNeasy Micro Kit (Qiagen). qPCRs were run on a Lightcycler 1.5 (Roche) using FastStart DNA master SYBR green I reagents, using KiTa (Qiagen). qPCRs were run on a Lightcycler 1.5 (Roche) using FastStart DNA master SYBR green I reagents, using kinetic PCR cycles. Offline analysis to calculate efficiency-controlled relative expression levels or absolute copy numbers was carried out according to Rasmussen (58). Intron-spanning primers were selected with Oligo 6.0 software (MedProbe) and PCR conditions, primer concentration and MgCl₂ concentration were optimized as described (55). Reactions were performed in glass capillaries in a volume of 20 µl. PCR products were analyzed by gel electrophoresis, melting curve analysis and control PCRs. Primers and PCR targets: mNaᵥ1.9 (AF118044): 1992-for 5′-CCCTTTGTGAGTCTCTGTCGAC-3′; 2114-rev 5′-GGAGTGGCCGATGATCTTAGAGCCGACAAATTGCCTAGC-3′; 141 bp, intron-spanning. mNaᵥ1.8 (NM_009134): 2541-for 5′-CATTCCCTTCCTCGTCGTC-3′; 2676-rev 5′-AAAGCGGATGAATTAGGTAAGG-3′; 155 bp, intron-spanning. mNaᵥ1.5 (NM_021544): 712-for 5′-TTCACCGCCATCTACACCT-3′; 855-rev 5′-GAAGCCGACAAATTGGCTAGC-3′; 163 bp, intron-spanning. GAPDH (NM_008048): 205-for 5′-GCAAAATTCAGGCA-3′; 337-rev 5′-CACCAGTAGACTCCACGAC-3′; 141 bp, pseudo-genes. β-Actin (NM_007393): for 5′-GCAACCGTGAAGATGAC-3′; rev 5′-GGCGTGAGGGAGAGCATAG-3′. Kinetic cycle conditions in four segments: mNaᵥ1.9 (95°C, 0 s, 59°C, 5 s, 72°C, 6 s, 83°C, 5 s), 2 mm MgCl₂, 30 pmol primer; efficiency (55). mNaᵥ1.8 (95°C, 0 s, 58°C, 5 s, 72°C, 7 s, 85°C, 5 s) 2 mm MgCl₂, 30 pmol primer. mNaᵥ1.5 (95°C, 0 s, 59°C, 5 s, 72°C, 7 s, 85°C, 5 s) 2 mm MgCl₂, 30 pmol primer. GAPDH (95°C, 0 s, 59°C, 5 s, 72°C, 6 s, 83°C, 5 s) 3 mm MgCl₂, 30 pmol primer; efficiency: 2.00. β-Actin (95°C, 10 s, 55°C, 10 s, 72°C, 5 s, 86°C, 5 s) 2 mm MgCl₂, 10 pmol primer.

**Laser capture microdissection**

Laser capture microdissection (Leica DM6000B laser microdissection system) was used to isolate motoneurons from the spinal cord of 2- and 4-day-old postnatal mice. In addition, DRG neurons, which express high numbers of *Naᵥ1.9* transcripts, were collected as reference. Spinal cord sections were embedded in optimum cutting temperature compound (Tissue-Tek) and immediately immersed in isopentane, chilled in liquid nitrogen for rapid freezing. Cross-sections of 15 µm thickness were prepared on a Leica cryostat, transferred to 0.9 µm POL membranes (Leica) and stained in Cresyl Violet solution. A total of 500–1200 MN cell bodies were collected in RNA lysis buffer. Total RNA was purified from the samples (59) and real-time RT–PCR was performed (see above). To compare expression levels of *Naᵥ1.9* in motoneurons versus DRG neurons, GAPDH expression served as denominator and relative expression was calculated.

**Cloning of mouse *Naᵥ1.9*, shRNA expression vectors**

For stable propagation in *E. coli*, the rop-element of pBR322 was introduced to the backbone of pcDNA3 as described earlier (33). Next, mouse *Naᵥ1.9* was cloned from mouse brain mRNA by fusing three partial cDNA PCR fragments. An artificial Kozak sequence (CCACCATG) was introduced before the start codon. The resulting construct was sequenced on both strands. Expression was tested by western blot analysis and immunocytochemistry using antibodies against *Naᵥ1.9* (71n) and pan-Naᵥ1.X (Sigma, clone K58/35, 1–2 ng/ml). For lentiviral expression of shRNA targeted against *Naᵥ1.9* transcripts and shRNA missense controls, two series of vectors were used. Sequences were selected (60) and cloned to the vector LL3.7 (61). In this case, shRNA was directed against the 3′ UTR of *Naᵥ1.9* and expressed under U6 promoter. GFP expressed under cytomegalovirus (CMV) promoter served as infection control. In a second set basing on pSIH-H1 (System Biosciences), we replaced CMV-driven copGFP against TDTOmato (62) and expressed shRNA against the coding region of *Naᵥ1.9* and corresponding missense control shRNA under H1 promoter. To test the shRNA performance, mNaᵥ1.9 expression backbone (A.W., S.H. and R.B., in prep) was mixed with shRNA expression vectors, transfected to HEK293T cells and co-expressed for 72 h. Then cells were lysed and Naᵥ1.9 protein was visualized by western blot analysis. In a second set of experiments, shRNA expression vectors were transfected to a cell with stable expression of mNaᵥ1.9 and quantitative analysis of shRNA efficiency was determined by western blot analysis.

**Antibody production: anti-mouse *Naᵥ1.9* (71n)**

First, pre-immune serum was tested by immunofluorescence and western blot analysis. Antibodies were raised in pre-tested
rabbits against the C-terminus of mouse Nav1.9. As antigen, a keyhole limpet hemocyanin-coupled peptide (CNGDLSSLDVPKIKVHCD) was used. Sera were tested after the fourth (71) and fifth boost (71n). Antibodies were affinity purified (immunization peptide on column) and stored in Tris–glycine, 250 mM NaCl, 1 mg/ml bovine serum albumin, 0.01% Thimerosal, 50% glycerol at −20°C at a final concentration of 400 μg/ml.

Detection of Nav1.9 in HEK293T cells (recombinant) or DRGs (endogenous) using western blot analysis

DRGs were prepared from adult Nav1.9−/− mice and wild-type mice of either sex, separated from nerve roots and collected in phosphate-buffered saline (PBS) on ice. Four to six lumbar or 6–10 thoracic DRGs were homogenized in 90 μl of 2% NP40 (or Igepal), 150 mM NaCl, 50 mM Tris–HCl, 10 mM DTT (fresh), 10% glycerol including protease inhibitors (Roche) using a ultrasonic homogenizer (HUESLER UP50H with MS1 sonotrode). After a first round of a 10 s pulse with 100% amplitude, lysates were incubated on ice for 10 min and homogenized again under same conditions. An aliquot of 75 μl of the sample were mixed with 4× Laemmli SDS–PAGE loading buffer and incubated at 50°C for 10 min. HEK293T protein was prepared in 2% NP40 (or Igepal), 150 mM NaCl, 50 mM Tris–HCl, 10% glycerol including protease inhibitors (Roche). SDS–PAGE on NaV1.9 was performed with Tris–Acetate gradient gels (4–7%; BioRad). Protein lysates (40–60 μg per lane) were blotted (semi-dry) on polyvinylidene difluoride membranes (BioRad), blocked with 5% milk powder (BioRad) and labeled with rabbit anti-Nav1.9 (71n) (1:1000) and re-probed with mouse anti-human γ-adaptin (1:2000; Sigma, clone 100/3) for loading control. As loading control for DRG protein, anti-TrkB (C14, Santa Cruz, 0.2 μg/ml) was used. Antibodies were detected using ECL-detection reagents (GE Healthcare).

Lentivirus production

In HEK293T cells, pSIH-H1-based lentiviral vectors were packaged with pCMV-HSV and pCMV4AR8.91 (63) as described earlier (64). shRNA expressing pL7.37 was packaged with pRSV Rev, pMDL/gpRRE and pMD.G (65,66). Vectors were transfected with Lipofectamine 2000 (Invitrogen) in OptiMEM medium with 10% fetal calf serum for 12–14 h and viral supernatants were harvested 72 h after transfection. Lentiviral particles were concentrated from clarified supernatants by two rounds of ultracentrifugation at 25 000 rpm in a Beckman SW28 rotor for 2 h at 4°C. The virus pellet was soaked on ice for a minimum of 4 h in 200 μl tris-buffered saline buffer (in mM): 130 NaCl, 10 KCl, 5 MgCl2 and 50 Tris–HCl, pH 7.8. Aliquots (10 μl) of the viral suspension were stored at −80°C. Titration was performed on HeLa cells and 105 infectious particles were used to infect 5000 freshly prepared E14 motoneurons in suspension before plating.

Immunocytochemistry

Cultures were fixed in 4% PBS-buffered paraformaldehyde at 37°C for 10–20 min. Blocking and permeabilization was performed in 1× PBS, 10% BSA, 0.05–0.1% Triton X-100, 0.1% Tween 20, pH 7.4, for 1 h. For visualization of Nav1.9 in axons, 0.3% Triton X-100 was used. Primary antibodies were incubated in blocking solution for 3 h at room temperature. The following antibodies were used: rabbit anti-Tau (Sigma, 1/1000); mouse anti-Map2 (clone AP-20, Sigma, 1/400); pan-anti-Sodium channel (Nav1.1-Nav1.6; Sigma, clone K585/35, 1/500); mouse anti-Tubulin (Sigma, clone B-5-1-2, 1/2000). In washing steps, 1× PBS, 0.1% Triton X-100, 0.1% Tween 20 was used. Samples were detected by secondary antibodies labeled with Alexa 488 (Invitrogen), Dylight 488, Cy3, or Cy5 (Jackson Immunoresearch). Nuclei were labeled by DAPI (400 pg ml−1) in PBS. Actin was labeled with Alexa Fluor-546 phalloidin (Invitrogen). Cells were embedded in Aqua Polymount (Polysciences) and monitored using Leica confocal systems (TCS SP series) equipped with Leica objectives (HC PL Apo ×20/0.7; HCX Apo ×40/1.25–0.75 oil, ×60/1.4–0.6 oil).

STED microscopy

STED microscopy was performed on a Leica SP5 confocal laser scanning microscope equipped with a Mai-Taï multi photon laser (Spectra-Physics). mNav1.9 (71n) immunoreactivity (ImmR) was detected with anti-rabbit Atto647N secondary antibodies. A Leica HCX PL Apo CS ×100/1.4 oil objective was used for the STED detection. Serial scanning of anti-Tubulin and phalloidin ImmR was performed by standard confocal imaging. Images were taken at 12-bit, in single confocal planes.

Axon length analysis

The axon length was determined by applying a morphometric system (Leica, Bensheim, Germany) on confocal image material.

Confocal Ca2+ imaging

A 5 mM stock solution of Oregon green-BAPTA1-AM (Invitrogen; O6807) was prepared in 8.9 μl of 20% Pluronic F-127 (Invitrogen) in dimethyl sulfoxide by means of a sonifier bath (Bandelin) for 2 min. Motoneurons were incubated with 5 μM Oregon green-BAPTA1-AM in ACSF solution. Motoneurons were loaded with dye-containing ACSF solution in a cell culture incubator (37°C, 5% CO2) for 10–15 min. Motoneurons were imaged at an inverted confocal microscope (Leica SP series) using a ×20/0.7 objective under continuous perfusion in a low chamber volume (~200 μl) with high buffer exchange rate (~10× chamber volume per min, in some experiments: ~20× volume exchange/min). Ligands (Sigma, Ascent Scientific) were used at the following concentrations: tetrodotoxin (TTX; 100–10 nm) and saxitoxin (STX; 100–10 nm). In some cases, muscimol (10–50 μM) or γ-aminobutyric acid (GABA) (100 μM) were used as control stimulus to monitor the excitatory action of GABA on embryonic motoneurons. Time lapse monitoring (256 × 256 pixel) of Ca2+ dynamics was performed at 2.0 Hz. Oregon green-BAPTA1-derived fluorescence was excited with a 488 nm laser line (emission detection: 507/565 nm). For pharmacological effect of VGSC inhibitors on spontaneous Ca2+ transients in motoneurons, cells were analyzed in their active state, under conditions of activity block.
and in a phase of the recovery of activity after pharmacological treatment (Fig. 3). In case of NaV1.9−/− motoneurons and strain-matched control cells (Fig. 8), all cells in the field of interest were analyzed. ACSF contained (in mM): 127 NaCl, 3 KCl, 2.5 NaH₂PO₄, 2 CaCl₂, 2 MgCl₂, 23 NaHCO₃ and 25 d-glucose, bubbled with 95% O₂/5% CO₂.

Image analysis
Images were analyzed using ImageJ software (WS Rasband, ImageJ, US National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/, 1997–2006) and processed with Photoshop (Adobe Systems, San Jose, CA, USA). XY-time Ca²⁺ imaging results were analyzed by a roi analysis (pixel intensity; Δf₀/₀ analysis).

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS
We are grateful to Hilde Troll, Regine Sendtner, Nicole Elflein and Michaela Kessler for excellent technical assistance and Mike Friedrich for introduction in STED microscopy. NaV1.9−/− mice were kindly provided by John Wood, London.

Conflict of Interest statement. None declared.

AUTHORS’ CONTRIBUTIONS
R.B., M.S. designed research; N.S., A.W., B.D., P.Y., S.H., S.J., R.B. performed research; M.A.N. contributed analytic tools; N.S., A.W., B.D., P.Y., S.J. and R.B. analyzed data; R.B. and M.S. wrote the paper.

FUNDING
This work was supported by grants from the Deutsche Forschungsgemeinschaft, BL567 and SFB581, project B1 and B24 and the from the Hermann und Lilly Schilling Stiftung im Stifterverband der Deutschen Wissenschaft. P.Y. was supported by a grant from the German Excellence Initiative to the Graduate School of Life Sciences, University of Würzburg.

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