Role of the terminal domains in sodium channel localization

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Key words: sodium channels, localization, neuron, axon, myelination

Voltage-gated sodium channels are membrane proteins that initiate action potentials in neurons following membrane depolarization. Members of this family show differential distribution at the subcellular level. The mechanisms underlying the targeting of these isoforms are not understood. However, their specificity is important because the isoforms can change the excitability of the membrane due to differences in their electrophysiological properties. In this study, chimeras generated between NaV1.2 and NaV1.6 were used to test channel domains for sequence that would allow NaV1.2 to localize to unmyelinated axons when NaV1.6 could not. We show that the N-terminal 202 amino acids of the NaV1.2 channel can mediate membrane domain-specific sorting in polarized epithelial cells and are necessary but not sufficient for localizing the isoform to the axons of cultured neurons. The domain-sorting signal is in the region between amino acids 110–202 of the NaV1.2 channel. The C-terminal 451 amino acids of NaV1.2 likely contain determinants that interact with neuron-specific factors to direct NaV1.2 to the axon.

Introduction

Voltage-gated sodium channels are responsible for the initiation phase of action potentials in neurons. There are nine mammalian isoforms of this channel, and they differ in their developmental and tissue-specific expression.1 NaV1.2 and NaV1.6 are two sodium channel isoforms expressed at high levels in the adult CNS. Although the isoforms are very similar in sequence, they differ in their electrophysiological properties and intracellular localization. NaV1.2 is found in unmyelinated axons of neurons in tissues and dissociated cultures.2-4 NaV1.2 also populates immature nodes of Ranvier in hypomyelinated axons, and it is completely replaced by NaV1.6 at mature nodes along compact myelinated axons.5,6 Given that both isoforms are expressed in some neurons,5,7,8 molecular differences between NaV1.2 and NaV1.6 may include a determinant that allows NaV1.2 to localize to the axonal domain in the absence of myelination.

In cells that express both NaV1.2 and NaV1.6 isoforms, the mRNA transcripts remain in the soma,7 indicating that the proteins are targeted post-translationally. Previous studies have suggested that the axon localization of NaV1.2 may be dependent on its recruitment by the domain-specific protein ankyrin G,9 or on increased endocytosis in the dendrites that effectively lowers the steady state presence of NaV1.2 in these regions.10 However, since the domain II-III linker sequence mediating ankyrin G-binding and the C-terminal di-leucine motif facilitating endocytosis are also present in NaV1.6, they cannot account for the difference in the isoforms’ distributions in nonmyelinating conditions.

NaV1.2 and NaV1.6 have the greatest sequence variance in their cytoplasmic termini and the I-II and II-III domain linker regions. In this study, we examined whether the terminal domains contribute to the NaV1.2 channel’s ability to localize in unmyelinated axons. We generated chimeric channels between NaV1.2 and NaV1.6, which is not normally found in unmyelinated axons, and examined axonal distribution of the channels. The channels were tagged with fluorescent proteins to allow direct visualization of their localization in cells. The results suggest that the terminal regions of NaV1.2 are involved in different steps of the axonal sorting process.

Results

Attachment of GFP or CFP at the N-terminus does not alter channel function. To visualize the heterologously expressed sodium channels and to distinguish them from native channels, the NaV1.2 and NaV1.6 isoforms were tagged at their amino-termini with variants of green fluorescent protein. NaV1.2 was tagged with enhanced green fluorescent protein (NaV1.2-EGFP) and NaV1.6 was tagged with enhanced cyan fluorescent protein (NaV1.6-ECFP). The tags were inserted at the end of the cytoplasmic N-terminus to minimize any effect the additional sequences might have on channel structure or localization.

To determine if the tags significantly altered the channel, electrophysiological properties of the tagged channels were compared to those of wild-type channels after expression in Xenopus oocytes. We used FLAG-tagged NaV1.2 as wild-type NaV1.2 because the FLAG epitope tag did not change the functional properties of the NaV1.2 channel.11 NaV1.2-EGFP showed similar voltage dependence of activation as NaV1.2-FLAG, with the small voltage
Figure 1. Characterization of Tagged NaV1.2 and NaV1.6. (A) **Filled symbols**: To determine the voltage dependence of activation, *Xenopus* oocytes expressing NaV1.2-FLAG or NaV1.2-EGFP were depolarized in 10 mV increments from -90 to +30 mV from a holding potential of -100 mV. Conductance values were calculated by dividing the peak current amplitude by the driving force at each potential and normalizing to the maximum conductance, as described in the Materials and Methods. Sample sizes and $V_{1/2}$ were 3 and -5.3 ± 0.6 mV for NaV1.2-FLAG, and 6 and -8.7 ± 3.5 mV for NaV1.2-EGFP. The values shown are averages and the error bars are standard deviations. **Open symbols**: To determine the voltage dependence of inactivation, *Xenopus* oocytes expressing NaV1.2-FLAG or NaV1.2-EGFP were depolarized from a holding potential of -100 mV to a range of conditioning potentials between -95 mV and +25 mV for 100 ms, followed immediately by a test pulse to -5 mV. The current amplitude during each test pulse was normalized to the peak current amplitude and plotted as a function of the conditioning pulse potential. Sample sizes and $V_{1/2}$ were 3 and -33.9 ± 0.8 mV for NaV1.2-FLAG, and 5 and -33.8 ± 1.6 mV for NaV1.2-EGFP. The values shown are averages and the error bars are standard deviations. (B) To determine the kinetics of fast inactivation, sodium current traces elicited by 100 ms depolarizations between -25 mV and +25 mV from a holding potential of -100 mV were fitted with the double exponential equation described in the Materials and Methods. Sample sizes were 3 for NaV1.2-FLAG and 4 for NaV1.2-EGFP. Time constants for the slow ($\tau_{\text{slow}}$, filled circle) and the fast ($\tau_{\text{fast}}$, open circle) components of fast inactivation are plotted on a logarithmic scale. The values shown are averages and the error bars indicate standard deviations. (C) Fraction of current inactivating with $\tau_{\text{fast}}$ as determined by the fits for NaV1.2-FLAG and NaV1.2-EGFP. (D) Voltage-dependent activation (filled squares) and inactivation (open squares) for NaV1.6 and NaV1.6-ECFP were determined as described in panel A. Sample sizes and $V_{1/2}$ of activation were 8 and -13.2 ± 3.7 mV for NaV1.6, and 5 and -10.1 ± 3.7 mV for NaV1.6-ECFP. Sample sizes and $V_{1/2}$ of inactivation were 3 and -61.0 ± 1.3 mV for NaV1.6, and 5 and -57.7 ± 3.2 mV for NaV1.6-ECFP. (E) Kinetics of fast inactivation for NaV1.6 and NaV1.6-ECFP were determined as described in (B). Sample sizes were 3 for NaV1.6 and 5 for NaV1.6-ECFP. Filled squares are $\tau_{\text{slow}}$ values, and open squares are $\tau_{\text{fast}}$ values. (F) Fraction of current inactivating with $\tau_{\text{fast}}$ as determined by the fits for NaV1.6 and NaV1.6-ECFP.
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of native Na\(_{\text{v}}\)1.2 and Na\(_{\text{v}}\)1.6 by immunofluorescent staining (Fig. 2A, green staining). Antibodies specific for the microtubule-associated protein MAP2 were used to distinguish the MAP2-containing somatodendritic domains from the MAP2-lacking axonal processes (Fig. 2, red staining). In 10-to-14-day cultures, Na\(_{\text{v}}\)1.2 staining was visible in all regions of the neuron, including the dendrites and soma that stained with MAP2 and the axon that did not stain with MAP2 (Fig. 2A, top row). In contrast, Na\(_{\text{v}}\)1.6 staining was present only in the dendrites and soma and not in the axon (Fig. 2A, bottom row). In neurons transfected with the tagged channels, Na\(_{\text{v}}\)1.2-EGFP was found most often in the soma and axon (Fig. 2B, top row), although in some cases the tagged channel was also present in the dendrites (Table 2). On the other hand, Na\(_{\text{v}}\)1.6-ECFP was observed in the soma and dendrites but not the axon (Fig. 2B, bottom row; Table 2). Thus, the tagged channels showed expression patterns similar to endogenous Na\(_{\text{v}}\)1.2 and Na\(_{\text{v}}\)1.6, so the tags did not appear to alter the channels’ localization properties.

Figure 2. Expression of Endogenous and Exogenous Na\(_{\text{v}}\)1.2 and Na\(_{\text{v}}\)1.6 in Neurons. (A) Mouse cortical neuroglial cultures were maintained for 14 days in vitro, fixed, permeabilized and stained with antibodies against MAP2 (red) and either Na\(_{\text{v}}\)1.2 or Na\(_{\text{v}}\)1.6 (green). Arrows indicate the axon. (B) Neuroglial cultures were transiently transfected with tagged sodium channels and examined for expression by immunofluorescence. Fluorescence localization showed that Na\(_{\text{v}}\)1.2-EGFP was present in the axons (arrow), but Na\(_{\text{v}}\)1.6-ECFP (cyan) was not. The axons were identified as the processes not stained with antibodies against MAP2 (red). Scale bars, 20 \(\mu\)m.

difference observed being statistically insignificant by t-test analysis (Fig. 1A, solid symbols). Na\(_{\text{v}}\)1.2-EGFP also showed similar voltage dependence of inactivation as Na\(_{\text{v}}\)1.2-FLAG (Fig. 1A, open symbols). The two constructs showed similar inactivation kinetics except at potentials -10 mV and -5 mV, where the fast component (\(\tau_{\text{fast}}\)) of inactivation rate was slightly slower for Na\(_{\text{v}}\)1.2-EGFP (Fig. 1B, open symbols), and at potentials -5 mV and 0 mV, where a higher fraction of the Na\(_{\text{v}}\)1.2-EGFP current inactivated with \(\tau_{\text{fast}}\) (Fig. 1C). Na\(_{\text{v}}\)1.6-ECFP and wild-type Na\(_{\text{v}}\)1.6 showed similar voltage dependence of activation (Fig. 1D, solid symbols), voltage dependence of inactivation (Fig. 1D, open symbols), and inactivation kinetics (Fig. 1E and F).

To determine whether the fluorescent protein tags affected the localization of the channels, we compared the cellular distribution of the tagged channels with the distribution of the endogenous channels in neurons. We used low-density, mixed cultures containing dissociated cerebral cortical neurons and glial cells to simulate conditions in which axons were unmyelinated. Isoform-specific antibodies were used to determine the distribution for the axonal localization of Na\(_{\text{v}}\)1.2. Terminal sequences were swapped between Na\(_{\text{v}}\)1.2 and Na\(_{\text{v}}\)1.6 to generate the chimeric channels shown in Figure 3. The regions that were swapped included the amino terminus up to I53, which will be referred to as the N-region, and the carboxy terminus plus domain IV, which will be referred to as the C-region. The chimeras were named based on the parental channel followed by the region that was substituted from the other isoform. For example, Na\(_{\text{v}}\)1.2-N6 consists of the Na\(_{\text{v}}\)1.2 channel with the Na\(_{\text{v}}\)1.6 N-region and Na\(_{\text{v}}\)1.2-C6 consists of the Na\(_{\text{v}}\)1.2 channel with the Na\(_{\text{v}}\)1.6 C-region. Comparable chimeras in which the terminal regions from Na\(_{\text{v}}\)1.2 were inserted in Na\(_{\text{v}}\)1.6 were also constructed (Na\(_{\text{v}}\)1.6-N2 and Na\(_{\text{v}}\)1.6-C2). All chimeras were tagged with variants of the green fluorescent protein at the N-terminus and verified to be functional in Xenopus oocytes.

Because of the low transfection efficiency in dissociated neuron cultures, we first tested if the terminal domains of the Na\(_{\text{v}}\)1.2 affected the channel localization process in cultures of polarized Madin-Darby canine kidney (MDCK) cells. MDCK cells have

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been used extensively for studying the post-translational targeting of a number of soluble and membrane-bound proteins because of the cell's ability to segregate proteins to different membrane domains separated by impermeable tight junctions.\textsuperscript{16,17} Localization in MDCK cells has been suggested to correlate with localization in neurons, with the MDCK basolateral domain corresponding to the neuronal somatodendritic region and the MDCK apical domain corresponding to the neuronal axon.\textsuperscript{16} For example, the K\textsubscript{s}2.1 voltage-gated potassium channel that localizes to the somatodendritic region in neurons sorts to the lateral membrane domain of MDCK cells.\textsuperscript{18,19}

We first examined the distribution of the parental Na\textsubscript{v}1.2-EGFP channel in transiently transfected MDCK cells. Fluorophore-conjugated wheat germ agglutinin (WGA) was added to the apical surface of tight MDCK cell monolayers to label the surface carbohydrate residues on the apical membrane. Because WGA could not penetrate the membrane or tight junctions between cells, it did not label the basolateral membrane. Thus, we were able to distinguish the apical membrane from the basolateral membrane of the MDCK cell. Approximately 47\% of the cells expressing Na\textsubscript{v}1.2-EGFP localized the channel to the apical membrane, which was marked by WGA binding (Fig. 4, row 1), to the exclusion of the basolateral membrane (Table 1). In contrast, transiently expressed Na\textsubscript{v}1.6-ECFP remained mostly cytoplasmic and was not observed in the WGA-labeled apical membrane (Fig. 4, row 2).

To verify that Na\textsubscript{v}1.2 was inserted into the apical membrane, a surface biotinylation assay was performed. MDCK cells stably expressing FLAG-tagged Na\textsubscript{v}1.2 or myc-tagged Na\textsubscript{v}1.6 were grown to a tight monolayer on permeable filters so that each of the polarized membrane domains could be accessed separately. The monolayer cultures were then biotinylated from either the apical or the basolateral surface to label only proteins inserted in the specific membrane domain. The tagged channel proteins were precipitated with antibody against the epitope tag, after which the biotinylated channel proteins were precipitated with streptavidin. Finally, the sodium channel proteins were visualized on western blots stained with tag-specific antibody. Na\textsubscript{v}1.2-FLAG was detected from the apical fraction but not the basolateral fraction (Fig. 5A), indicating that this channel was inserted into the apical membrane of MDCK cells. In contrast, Na\textsubscript{v}1.6-myc was not detected in either the apical or basolateral fraction (Fig. 5B), although it was visualized in the cells by immunofluorescent staining (Fig. 5C). Na\textsubscript{v}1.6-myc expression was noticeably absent from cell-to-cell membrane junctions. These results indicate that Na\textsubscript{v}1.6 remained in the cytoplasm of MDCK cells.

Because the biotinylation assay results supported the fluorescence localization data, it seemed reasonable to conclude that the domain distributions of the channel chimeras could be interpreted from similar fluorescence localization experiments. When the N-terminal 202 amino acids of Na\textsubscript{v}1.2 were replaced with the corresponding domain from Na\textsubscript{v}1.6 (Fig. 3, Na\textsubscript{v}1.2N6-ECFP), the chimera showed either cytoplasmic expression or accumulation around the basolateral domain but did not appear in the apical domain (Fig. 4, row 3), suggesting that the N-region of Na\textsubscript{v}1.2 was necessary for apical sorting. In contrast, when the C-terminal 451 amino acids of Na\textsubscript{v}1.2 were replaced with the corresponding domain from Na\textsubscript{v}1.6 (Fig. 3, Na\textsubscript{v}1.2C6-EGFP), the chimera was localized to the apical domain like the parental Na\textsubscript{v}1.2 channel (Fig. 4, row 4), indicating that the C-region of Na\textsubscript{v}1.2 was not necessary for apical localization. When the N-region of Na\textsubscript{v}1.2 was inserted in Na\textsubscript{v}1.6 (Fig. 3, Na\textsubscript{v}1.6N2-ECFP), the chimera showed either cytoplasmic expression or accumulation around the basolateral domain but did not appear in the apical domain (Fig. 4, row 5), suggesting that the N-region was sufficient for apical domain sorting. Swapping the C-region of Na\textsubscript{v}1.2 into Na\textsubscript{v}1.6 (Na\textsubscript{v}1.6C2-ECFP) did not localize the Na\textsubscript{v}1.6 channel in the apical domain (Fig. 4, row 6). These results indicate that the N-terminal 202 amino acids of Na\textsubscript{v}1.2 are important for the sodium channel localization process in MDCK cells.

The N-region can be further divided into two distinct domains: a cytoplasmic N-terminus domain and a mainly transmembrane DIS1-S3 domain. To determine which part of the N-region is...
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Figure 4. Chimeric Channel Localization in MDCK Cells. Representative micrographs of the localization of fluorescent protein-tagged chimeras in MDCK cells are shown. Apical membrane domain was defined by wheat germ agglutinin (WGA) labeling of apical surface glycoproteins on nonpermeabilized MDCK cells in polarized monolayer culture. (A) Phase contrast image of MDCK cells. (B) WGA labeling of the apical membrane domain. (C) WGA staining in the x-y plane imaged at 2.1–3.9 μm below the apical membrane domain established in (B). (D) Fluorescence localization of chimera at the apical membrane domain. (E) Fluorescence localization of chimera at the same x-y plane as (C). Scale bars, 10 μm.

Discussion

We have shown that Na\textsubscript{v}1.2, but not Na\textsubscript{v}1.6, is able to localize to the axonal domain of neurons cultured under nonmyelinating conditions. Na\textsubscript{v}1.2 also sorts to the apical membrane domain in MDCK cells, which have been used to model the polarized sorting of neuronal proteins such as voltage-gated potassium channels.\textsuperscript{18} Both the N- and C-regions of Na\textsubscript{v}1.2 are necessary for axonal localization in neurons, but only the N-region is necessary and sufficient for apical localization.

Multiple domains of Na\textsubscript{v}1.2 affect axonal localization. Since the apical membrane domain of MDCK cells appears to correlate with the axonal domain of neurons, we hypothesized that the N-region of Na\textsubscript{v}1.2 might also be involved in axonal localization. When the N-region chimeras were examined in neurons, Na\textsubscript{v}1.2N6-ECFP clustered in the somatodendritic region but not in the axon (Fig. 6, row 1; Table 2). Na\textsubscript{v}1.6N2-ECFP also remained only in the somatodendritic region (Fig. 6, row 3; Table 2). Therefore, the N-region of Na\textsubscript{v}1.2 was necessary but not sufficient to confer axonal localization to the Na\textsubscript{v}1.6 sodium channel. This result suggests that other regions of the channel are also involved in the localization process. Although the C-region of Na\textsubscript{v}1.2 was shown to be unnecessary for mediating the apical sorting of the channel in MDCK cells, it was possible that it was important for neuronal localization. Both Na\textsubscript{v}1.2C6-EGFP and Na\textsubscript{v}1.6C2-ECFP were only visible in the somatodendritic region (Fig. 6, rows 2 and 4; Table 2), suggesting that the C-region of Na\textsubscript{v}1.2 was also necessary but not sufficient to mediate axonal localization. The observation that Na\textsubscript{v}1.2 with the Na\textsubscript{v}1.6 C-region was somatodendritic indicates that the C-region is important for axonal localization in neurons, even though it was not necessary for apical localization in MDCK cells. Preliminary analysis of a chimera in which both the N- and C-regions of Na\textsubscript{v}1.2 were swapped into the corresponding regions of Na\textsubscript{v}1.6 showed that the chimera was present in the neuronal axon (Fig. 6, row 5), suggesting that the N- and C-regions of Na\textsubscript{v}1.2 might work together to effect axonal localization.

 responsibly for its role in Na\textsubscript{v}1.2 localization, chimeras that specifically switched either the N-terminus (amino acids 1–109) or the DIS1-S3 domain (amino acids 110–202) between Na\textsubscript{v}1.2 and Na\textsubscript{v}1.6 were generated. The Na\textsubscript{v}1.6nt2-ECFP chimera, which is the Na\textsubscript{v}1.6 channel with its cytoplasmic N-terminus replaced by the corresponding region from Na\textsubscript{v}1.2, did not localize to the apical domain of MDCK cells (Fig. 4, row 7). In contrast, Na\textsubscript{v}1.6ts2-ECFP, which is the Na\textsubscript{v}1.6 channel with its DIS1-S3 domain replaced by the corresponding region of Na\textsubscript{v}1.2, showed apical domain sorting (Fig. 4, row 8). The reciprocal swap (Na\textsubscript{v}1.2ts6-ECFP) was sequestered away from the apical membrane in intracellular clusters (Fig. 4, row 9). Thus, apical sorting was mediated by the Na\textsubscript{v}1.2 sequence spanning amino acids 110–202.
in MDCK cells. These data indicate that there are similarities in the sorting pathways between neurons and MDCK cells, but that some factors are unique to each cell type.

**NaV1.2 is directly targeted to the neuronal axon.** At least two distinct sorting pathways could lead to axonal localization of NaV1.2. One possibility is that NaV1.2 is transported in vesicles that move directly into the axon. Alternatively, NaV1.2 could be specifically retained only in the axon after being transported in vesicles to both the axon and dendrites. Previously, it was shown that an internalization signal in the NaV1.2 C-terminus was able to enhance the endocytosis of the C-terminus polypeptide from neuronal membrane except in the axon, where it was likely retained by interaction with an axonal binding partner.10 The authors proposed that the compartmentalization of NaV1.2 could be the result of nonpolarized distribution of the channel followed by a selective elimination of the channel from the dendrites. However, our results suggest that the full-length NaV1.2 follows the direct targeting pathway because four of the six neurons expressing NaV1.2-EGFP in the axon did not show any detectable fluorescence in their dendrites. If transport vesicles were nonspecific in their domain targeting, then NaV1.2-EGFP should have also clustered in dendritic vesicles in these neurons. The two neurons that displayed both axonal and dendritic fluorescence demonstrated diffuse NaV1.2-EGFP expression in all of the processes. The fluorescence intensity in those neurons was higher than that in the neurons with somato-axonal expression, probably reflecting higher levels of NaV1.2-EGFP protein. It is likely that the NaV1.2 channel was overexpressed in those cells, resulting in nonspecific distribution throughout the neuron.

**Terminal regions of NaV1.2 mediate different steps in channel localization process.** Our results indicate that the terminal regions of NaV1.2 contribute to the subcellular localization of this protein. The loss of its native N-region prevents NaV1.2 from entering the apical membrane domain of polarized MDCK cells or the axons of neurons, which suggests that the N-region affects channel sorting through a mechanism common to the two cell types. Membrane proteins are normally synthesized in the endoplasmic reticulum (ER), modified in the Golgi complex, and distributed by transport vesicles. Given that NaV1.2N6-ECFP is shown to accumulate largely in the soma, we suspect that the chimera might be hindered on its way out to the peripheral processes.

One possibility is that the chimera is retained in the ER. NaV1.2 contains a potential retention signal (RKR) in its domain I-II linker, which has been shown to be active in regulating the export of K<sub>ATP</sub> potassium channels from the ER.20 Zhang et al. recently identified an ER retention signal with a similar RXR motif in the I-II linker of the NaV1.8 isoform that regulates the surface expression of the channel.21 The NaV1.2 N-region may have a role in masking this retention signal, and the homologous region from NaV1.6 may be less effective in this role. We have shown

### Table 1 Percentage of MDCK cells showing polarized domain distribution of tagged channel chimeras

|        | Apical | Baso-lateral | Cytoplasmic only | Everywhere |
|--------|--------|--------------|------------------|------------|
| NaV1.2-EGFP<sup>b</sup> | 47%   | 5%           | 26%              | 21%        | 38  |
| NaV1.6-ECFP<sup>c</sup> | 0%    | 10%          | 90%              | 0%         | 10  |
| NaV1.2N6-ECFP<sup>c</sup> | 0%   | 33%          | 67%              | 0%         | 6   |
| NaV1.2C6-EGFP<sup>b</sup> | 83%  | 0%           | 17%              | 0%         | 6   |
| NaV1.6N2-ECFP | 23%   | 0%           | 77%              | 0%         | 13  |
| NaV1.6C2-ECFP<sup>c</sup> | 0%   | 50%          | 33%              | 17%        | 6   |
| NaV1.6nt2-ECFP<sup>c</sup> | 0%    | 33%          | 67%              | 0%         | 6   |
| NaV1.6ts2-ECFP | 27%    | 0%           | 73%              | 0%         | 11  |
| NaV1.2ts6-ECFP<sup>c</sup> | 0%   | 0%           | 100%             | 0%         | 5   |

<sup>a</sup>n denotes the total number of transfected cells examined. <sup>b</sup>Apical versus basolateral + cytoplasmic distribution was statistically different compared to NaV1.6 (p < 0.02). <sup>c</sup>Apical versus basolateral + cytoplasmic distribution was statistically different compared to NaV1.2 (p < 0.02).

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**Figure 5. NaV1.2 Sorts to the Apical Membrane of MDCK Cells.** (A) NaV1.2-FLAG proteins expressed in the plasma membrane were isolated from the lysates of apically or basolaterally biotinylated MDCK cells stably expressing NaV1.2-FLAG. NaV1.2-FLAG proteins were first collected using antibodies against the FLAG epitope, and then the biotinylated NaV1.2-FLAG proteins were precipitated from the NaV1.2-FLAG pool using streptavidin. The final isolate was analyzed by SDS-PAGE and western blot using anti-FLAG antibody. Isolate from 2.7 x 10<sup>5</sup> cells was loaded in each lane. (B) NaV1.6-myc proteins were similarly collected from MDCK cells stably expressing NaV1.6-myc and analyzed, except anti-myc antibody was used in place of the anti-FLAG antibody. Isolate from 1.1 x 10<sup>6</sup> cells was loaded in each lane. (C) MDCK cells stably expressing NaV1.6-myc were fixed, permeabilized and probed with anti-myc antibody. Myc staining was absent from cell-to-cell membrane junctions (arrowheads). Scale bars, 10 μm.
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Attract the retention signal, and that motif is absent in the corresponding loop from NaV1.6. Alternatively, the DIS1-S3 sequence may contain a signal involved in vesicle selection in the trans Golgi network. Distinct apical and basolateral transport vesicles are involved in sorting membrane proteins in MDCK cells, and such a separation of domain-specific cargos may also underlie the targeting in neurons. The fact that single-swap chimeras show some dendritic distribution like NaV1.6-ECFP and not axonal distribution like NaV1.2-EGFP suggests the possibility of a default dendritic pathway for channels that are unable to access the axonal pathway. Examples of sorting signals residing in transmembrane sequence domains have been reported in the influenza virus proteins neuraminidase and hemagglutinin.

Loss of its native C-region prevents NaV1.2 from entering the neuronal axon but not the MDCK apical domain, which indicates that the C-region influences channel localization only in the neuron. The C-region of NaV1.2 may interact with a neuron-specific factor to move NaV1.2 into the axon. One attractive candidate is the sodium channel β2 subunit, which has been shown to associate with intracellular transport vesicles, to mediate the cell surface appearance of sodium channels from intracellular pools, and to cluster with NaV1.2 in unmyelinated axons. Given that NaV1.2 is known to bind the β2 subunit through a disulfide interaction, we speculate that if β2 exerts its localization effect through the C-region of NaV1.2, then it likely binds the C-region through cysteine residues in the extracellular S5-S6 loop of domain IV to shield the disulfide linkage from the reducing environment of the cytosol. Further analysis of the C-region is needed to define the actual effector sequence and its interaction with the localization machinery.

Materials and Methods

Channel constructs. The NaV1.2-FLAG construct was previously characterized. Briefly, the Scn2a cDNA encoding the rat NaV1.2 channel was tagged at the 5′ end with the synthetic FLAG sequence 5′-GAC TAT AAA GAC GAT GAC GAT AAA-3′. The construct was placed behind a cytomegalovirus (CMV) promoter for expression in mammalian cells and a T7 promoter to facilitate in vitro transcription.

To generate NaV1.2-EGFP, the EGFP sequence between NheI and BspEI was cut from the pEGFP-C1 plasmid (Clontech Laboratories, Palo Alto, CA), and the construct was inserted in frame into the 5′ noncoding sequence of the NaV1.2-FLAG clone behind the promoters and between Nhel and BspEI sites created
with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Primers used to create the Nhel site were 5’-GAG ACC GGA AGC TAG CTA CCG AGC TCC GGA GG-3’ and 5’-CCT CCG GAC CAG CATG CCT CCG GTC TC-3’, and primers used to create the BspEI site were 5’-GTT ACT CTC CGG AGG TCC ACT AGT AAC-3’ and 5’-GTT ACT GGA CCT CCG GAT CTA-3’. In addition, one intentional E937Q substitution was introduced that made the channel resistant to tetrodotoxin.13 The tagged and mutated full-length Scn8a cDNA sequence was excised from its vector plasmid with the XhoI and NotI restriction enzymes, ligated to a NotI-AatII-XhoI linker sequence, and inserted into a NotI site in the MCS of the mammalian vector pRC/CMV(A). The construct was named Scn8pCMV.

To generate NaV1.6-ECFP, the Scn8pCMV sequence between the SnaBI site in the CMV promoter and a Sall site in the 5’ noncoding region was exchanged with the corresponding sequence from the mutated NaV1.2-FLAG clone containing Nhel and BspEI sites, and then the ECFP sequence was cut from the pECFP-C1 plasmid (Clontech Laboratories) and inserted between the new restriction sites. Amino acid mutations at the positions 5, 15, 142 and 153 were reverted to wild-type residues using the QuikChange site-directed mutagenesis kit. An early termination codon in the linker between ECFP and NaV1.6 was mutated to a leucine residue using the following primers: (sense) 5’-GCG TCG ACG GTA CTC ATC TCA GAA GAG GAT CTA-3’ and (antisense) 5’-GTT ACT GGA AGC TAG CTA CCG AGC TCC GGA GG-3’ and 5’-CCT CCG GAC CAG CATG CCT CCG GTC TC-3’.

Chimeric constructs between the Scn2a and Scn8a cDNA’s were created using restriction sites shared between the two sequences. To generate the channel chimeras NaV1.6N2 and NaV1.2N6, the gene fragment before the Ndel restriction site (amino acid positions A202-Y203) in Scn2a was exchanged with the corresponding fragment from Scn8a. The resulting N-terminal chimeras switched channel isoform in the third transmembrane segment of domain I. To obtain the chimeras NaV1.2C6 and NaV1.6C2, the gene fragment after the BasEI restriction site (amino acid positions M1545-V1546) in Scn2a was exchanged with the corresponding fragment from Scn8a. The resulting C-terminal chimeras switched channel isoform in the first transmembrane segment of domain IV. To generate the channel chimera NaV1.6N2, the gene fragment before the BorGI restriction site (amino acid positions L112-Y113) in Scn8a was replaced with the corresponding fragment from Scn2a. The N-terminus of the resulting chimera came from NaV1.2, while the rest of the channel came from NaV1.6. To generate the chimeras NaV1.6ts6 and NaV1.6ts2, the gene fragment between the BorGI restriction site (amino acid positions L112-Y113) and the Ndel restriction site (amino acid positions A202-Y203) in Scn2a was exchanged with the corresponding fragment from Scn8a. Thus, the sequence spanning the first 2½ transmembrane segments and associated linkers in domain I of the chimera came from one isoform while the rest of the channel came from the other. Chimeric constructs were tagged, mutated and inserted into the pRC/CMV(A) plasmid as described for the parental constructs.

Cell cultures and transfection. MDCK cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 0.3 mg/ml L-glutamine, 100 units/ml penicillin G sodium and 100 μg/ml streptomycin sulfate. Transfection with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was performed following the manufacturer’s instructions on 80% confluent cultures. Transiently transfected cultures were processed for immunofluorescence three days past cell confluency.

To generate stably expressing cell lines, transfected cultures were maintained in 1 mg/ml G418 (Sigma Aldrich Corp., St. Louis, MO) selection for two weeks until the non-transfected control culture was cleared. The remaining colonies were subsequently expanded in 0.4 mg/ml G418.

Neuroglial mixed cultures were prepared from postnatal (P2-P3) mouse cortices as previously described,14 with modifications. Cells were dissociated by trituration in Neurobasal-A medium (Gibco-Invitrogen, Carlsbad, CA) supplemented with B27 (Gibco-Invitrogen), plated on poly-D-lysine-coated glass coverslips, and maintained with glial-conditioned Neurobasal-A medium supplemented with B27 and 0.5 mM glutamine. Half of the medium was replaced every three days. Seven to ten days after plating, cultures were transfected using Effectene (Qiagen, Valencia, CA) following the manufacturer’s instructions. Cultures were processed for immunofluorescence three days after transfection.

Glia1 cultures were prepared from postnatal (P2-P5) mouse cerebral cortices following the protocol for dissociated neuroglial co-cultures, but the cells were maintained in Modified Eagle’s Medium supplemented with 20 mM glucose, 10% fetal bovine serum, 50 units/ml penicillin G sodium, and 50 μg/ml streptomycin sulfate. Glial cultures were allowed to mature for two weeks before being used to condition the Neurobasal-A medium.

Expression and electrophysiology. RNA transcripts were generated using the T7 mMESSAGE mMACHINE transcription kit (Ambion, Austin, TX), and the yield was estimated by glyoxal gel electrophoresis. Stage V oocytes were prepared from adult *Xenopus laevis* as previously described.15 Approximately 0.2–2 ng of RNA

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### Table 2: Percentage of neurons showing polarized domain distribution of tagged channel chimeras

|                      | Somato-axonal | Somatodendritic | Somatic only | Everywhere | n^a |
|----------------------|---------------|-----------------|--------------|------------|-----|
| NaV1.2-ECFP          | 44%           | 11%             | 22%          | 22%        | 9   |
| NaV1.6-ECFP          | 0%            | 40%             | 60%          | 0%         | 5   |
| NaV1.2N6-ECFP        | 0%            | 33%             | 67%          | 0%         | 3   |
| NaV1.2C6-ECFP        | 0%            | 69%             | 31%          | 0%         | 13  |
| NaV1.6N2-ECFP        | 0%            | 100%            | 0%           | 0%         | 7   |
| NaV1.6C2-ECFP        | 0%            | 100%            | 0%           | 0%         | 3   |

^a^ Denotes the total number of transfected neurons examined. Somatoaxonal versus somatodendritic distribution was statistically different compared to NaV1.2 (p < 0.02).
were injected per oocyte to obtain current levels between 1 and 5 μA after 24–48 hours. Injected oocytes were incubated in ND-96 solution containing 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 0.1 mg/ml gentamicin, 0.55 mg/ml pyruvate, 0.5 mM theophylline and 5 mM HEPES at pH 7.5 for 24–72 hours at 20°C before analysis.

Sodium currents were recorded from oocytes at room temperature using the two-electrode voltage clamp OC-725 (Warner Instruments, Hamden, CT) with DigiData 1320A interface (Molecular Devices, Sunnyvale, CA) and pCLAMP 8 software (Molecular Devices). Oocytes were maintained in ND-96 solution (without gentamicin, pyruvate and theophylline) during recording. Transient capacitance and leak currents were corrected by P/4 subtraction or by subtraction of currents recorded in the presence of 400 nM tetrodotoxin.

The voltage dependence of activation was determined from the sodium currents elicited when the oocyte was step-depolarized from a holding potential of -100 mV to +30 mV in increments of 10 mV. Conductance (G) was calculated using the equation

\[ G = \frac{I}{(V - V_r)} \]

in which \( I \) is the current amplitude, \( V \) is the test potential, and \( V_r \) is the reversal potential. The reversal potential was extrapolated from fitting the I-V curve with the equation

\[ I = \frac{1}{1 + \exp(-0.03937 \times z \times (V - V_{1/2}))} \times g \times (V - V_r) \]

in which \( z \) is the gating charge, \( V_{1/2} \) is the half-maximal activity potential, and \( g \) is a factor related to the number of channels contributing to the observed current. Conductance values were normalized to the peak conductance and fitted with the two-state Boltzmann equation

\[ G = \frac{I}{1 + \exp[-0.03937 \times z \times (V - V_{1/2})]} \]

The voltage dependence of inactivation was determined from the sodium currents elicited during a -5 mV test pulse immediately after the oocyte was depolarized from a holding potential of -100 mV to a range of conditioning potentials between -95 mV and +25 mV for 100 ms. Currents from the test pulses were normalized to the peak current and fitted with the two-state Boltzmann equation

\[ I = I/[1 + \exp((V - V_{1/2})/a)] \]

in which \( I \) is the test pulse current, \( V \) is the conditioning potential, \( V_{1/2} \) is the half-maximal inactivation potential, and \( a \) is the slope factor.

The kinetics of fast inactivation were determined from the inactivation phase of the current traces elicited during the conditioning pre-pulses in the two-step inactivation protocol. The traces were fitted with the double-exponential equation

\[ I = A_{fast} \times \exp[-(t - K)/\tau_{fast}] + A_{slow} \times \exp[-(t - K)/\tau_{slow}] + C, \]

in which \( I \) is the current, \( A_{fast} \) and \( A_{slow} \) are the current fraction inactivating with the time constants \( \tau_{fast} \) and \( \tau_{slow} \), \( K \) is the time shift, and \( C \) is the steady-state non-inactivating current. The time shift was selected as the point at which the current trace began to inactivate exponentially.

**Immunofluorescence localization.** MDCK cells were fixed with 4% paraformaldehyde, blocked with 5% bovine serum albumin (BSA), and apically labeled with AlexaFluor 596-conjugated wheat germ agglutinin (Molecular Probes, Eugene, OR). Neuroglial cocultures were fixed with paraformaldehyde, permeabilized with 0.1% saponin, and blocked with BSA. MDCK cells stably expressing NaV1.6-myc were fixed with paraformaldehyde, permeabilized with 0.3% Triton X-100, and blocked with BSA. Primary antibodies used for neuron assays included anti-microtubule associated protein (MAP2) mouse IgG (Sigma-Aldrich Corp.), polyclonal rabbit antibody against NaV1.2 (Alomone Labs, Jerusalem, Israel), and polyclonal rabbit antibody against NaV1.6 (Chemicon International, Temecula, CA). The primary antibody used in MDCK cells expressing NaV1.6-myc was anti-c-myc mouse antibody (Roche Diagnostics Corp., Indianapolis, IN). Secondary antibodies included goat anti-mouse IgG-Alexa Fluor 596 (Molecular Probes), goat anti-rabbit IgG-Alexa Fluor 488 (Molecular Probe), sheep anti-mouse IgG-biotin (The Binding Site, Birmingham, United Kingdom), and streptavidin-fluorescein (Amersham Life Science, Buckinghamshire, United Kingdom). Immunostained cells were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and analyzed using a Zeiss Axiovert 200M microscope and Axiosvision software (Carl Zeiss North America, Thornwood, NY). Micrographs were processed with Axiosvision’s inverted filter deconvolution algorithm and Adobe Photoshop (Adobe Systems, San Jose, CA).

For analysis purposes, cellular domains were defined by labeling specific cell markers. The dendritic marker MAP2 was used in neurons for identifying the somal and dendritic domains. MAP2-lacking processes were regarded as axons. For MDCK cells, directionally applied wheat germ agglutinin (WGA) was used to distinguish the apical cell membrane from the unlabeled basolateral membrane. Sodium channels were considered to be in the apical cell domain if their fluorescence was coplanar with WGA staining. Channels were considered to be in the basolateral domain if their fluorescence traced the cell perimeter in horizontal sections of the cell below the WGA staining. Otherwise, fluorescence in the extranuclear space below the WGA staining was considered to represent cytoplasmic channel proteins. Statistical significance was determined by comparing 2 x 2 contingency tables with the Fisher Exact test using SigmaPlot 11 (Systat Software, San Jose, CA).

**Surface biotinylation and immunoprecipitation.** MDCK cells stably expressing NaV1.2-FLAG or NaV1.6-myc were grown to three days past cell confluency on polycarbonate permeable filters in Costar Transwell cluster plates (Corning-Costar, Corning, NY). Sulfo-NHS-SS-Biotin (Pierce Biotechnology, Rockford, IL) was applied from either the apical or the basolateral side of the cell monolayer according to the manufacturer’s instructions. Cells were lysed in a buffer containing 50 mM Tris at pH 7.5, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 1 μg/ml pepstatin A, 1 μg/
ml leupeptin, 2 μg/ml aprotinin, 1 mM PMSE, 0.1 mg/ml benzamidine and 8 μg/ml each of calpain I and calpain II. Cell debris was discarded, and the total lysate was subjected to immunoprecipitation with anti-c-myc mouse antibody (Roche Diagnostics Corp.,) or anti-FLAG mouse M2 antibody (Eastman Kodak, New Haven, CT) bound to a rabbit antibody against mouse IgG (Pierce Biotechnology) conjugated to Protein A-Sepharose CL-4B beads (Amersham Biosciences, Uppsala, Sweden). Biotinylated polypeptides eluted from this step were recaptured with Immobilized Streptavidin (Pierce Biotechnology). The final isolate was fractionated by SDS-PAGE, transferred onto nitrocellulose membranes, probed with the anti-c-myc or anti-FLAG antibody, and detected with HRP-conjugated antibody against mouse IgG (Pierce Biotechnology) and SuperSignal West Dura Substrate (Pierce Biotechnology).

Acknowledgements
This work was supported by grants from the National Multiple Sclerosis Society (RG3405A) and NIH (NS48336).

References
1. Goldin AL, Barchi RL, Caldwell JH, Hofmann F, Howe JR, Hunter JC, et al. Neuronal expression of voltage-gated sodium channels. Neuron 2000; 28:365-8.
2. Giraud P, Alcaraz G, Jullien F, Sampo B, Jover E, Couraud F, et al. Multiple pathways regulate the expression of genes encoding sodium channel subunits in developing neurons. Mol Brain Res 1998; 56:238-55.
3. Boiko T, Van Wart A, Caldwell JH, Levinson SR, Trimmer JS, Matthews G. Functional specialization of the axon initial segment by isoform-specific sodium channel targeting. J Neurosci 2003; 23:3306-13.
4. Garrido JJ, Fernandes F, Moussif A, Fache M-P, Giraud P, Dargent B. Dynamic compartmentalization of the voltage-gated sodium channels in axons. Biol Cell 2003; 95:437-45.
5. Boiko T, Rashand MN, Levinson SR, Caldwell JH, Mandel G, Trimmer JS, et al. Compact myelin dictates the differential targeting of two sodium channel isoforms in the same axon. Neuron 2001; 30:91-104.
6. Kaplan MR, Cho M-H, Ullian EM, Iosom LL, Levinson SR, Barres BA. Differential control of clustering of the sodium channels Na\textsubscript{v}1.2 and Na\textsubscript{v}1.6 at developing CNS nodes of Ranvier. Neuron 2001; 30:105-19.
7. Fjell J, Dib-Hajj S, Fried K, Black JA, Waxman SG. Differential expression of sodium channel genes in retinal ganglion cells. Mol Brain Res 1997; 50:197-204.
8. Oosnoo N, Alcaraz G, Padilla E, Couraud F, Delmas P, Crest M. Differential targeting and functional specialization of sodium channels in cultured cerebellar granule cells. J Physiol (Lond) 2005; 569:801-16.
9. Lemaillet G, Walker B, Lambert S. Identification of a conserved ankyrin-binding motif in the family of sodium channel subunits. J Biol Chem 2003; 278:27333-9.
10. Garrido JJ, Fernandes F, Giraud P, Moussif A, Fache M-P, Paquelin E, Fache M-P, et al. Identification of an axonal determinant in the C-terminus of the sodium channel Na\textsubscript{v}1.2. EMBO J 2001; 20:5990-61.
11. Smith RD, Goldin AL. Phosphorylation at a single site in the rat brain sodium channel is necessary and sufficient for current reduction by PKA. J Neurosci 1997; 17:6086-93.
12. Smith MR, Smith RD, Plummer NW, Meisler MH, Goldin AL. Functional analysis of the mouse Scn8a sodium channel. J Neurosci 1998; 18:6093-102.
13. Kontis KJ, Goldin AL. Site-directed mutagenesis of the putative pore region of the rat IIA sodium channel. Mol Pharmacol 1993; 43:635-44.
14. Li Z, Masengill JL, O'Dowd DK, Smith MA. Agrin gene expression in mouse somatosensory cortical neurons during development in vivo and in cell culture. Neuroscience 1997; 79:191-201.
15. Goldin AL. Expression of ion channels by injection of mRNA into Xenopus oocytes. Methods Cell Biol 1991; 36:487-509.
16. Dotti CG, Simons K. Polarized sorting of viral glycoproteins to the axon and dendrites of hippocampal neurons in culture. Cell 1990; 62:63-72.
17. Hasegawa J, Matsuzaki T, Tajika Y, Ablimit A, Suzuki T, Aoki T, et al. Differential localization of aquaporin-2 and glucose transporter 4 in polarized MDCK cells. Histochem Cell Biol 2007; 127:233-41.
18. Scannevin RH, Murakoshi H, Rhodes KJ, Trimmer JS. Identification of a cytoplasmic domain important in the polarized expression and clustering of the K\textsubscript{v}2.1 K\textsuperscript{+} channel. J Cell Biol 1996; 135:1619-32.
19. Lim ST, Antonucci DE, Scannevin RH, Trimmer JS. A novel targeting signal for proximal clustering of the K\textsubscript{v}2.1 K\textsuperscript{+} channel in hippocampal neurons. Neuron 2000; 25:385-97.
20. Zerangue N, Schwappach B, Jan YM, Jan LY. A new ER trafficking signal regulates the subunit stoichiometry of plasma membrane K\textsubscript{ATP} channels. Neuron 1999; 22:537-48.