Ankyrin G restricts ion channel diffusion at the axonal initial segment before the establishment of the diffusion barrier

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In mammalian neurons, the precise accumulation of sodium channels at the axonal initial segment (AIS) ensures action potential initiation. This accumulation precedes the immobilization of membrane proteins and lipids by a diffusion barrier at the AIS. Using single-particle tracking, we measured the mobility of a chimeric ion channel bearing the ankyrin-binding motif of the Nav1.2 sodium channel. We found that ankyrin G (ankG) limits membrane diffusion of ion channels when coexpressed in neuroblastoma cells. Site-directed mutants with decreased affinity for ankG exhibit increased diffusion speeds. In immature hippocampal neurons, we demonstrated that ion channel immobilization by ankG is regulated by protein kinase CK2 and occurs as soon as ankG accumulates at the AIS of elongating axons. Once the diffusion barrier is formed, ankG is still required to stabilize ion channels. In conclusion, our findings indicate that specific binding to ankG constitutes the initial step for Nav channel immobilization at the AIS membrane and precedes the establishment of the diffusion barrier.

Introduction

In mammalian neurons, the axonal initial segment (AIS) is a unique domain that plays a crucial role in the physiology of the nerve cell, as it orchestrates the interplay between electrogenesis and neuronal polarity. At the AIS, the concentration of voltage-gated ion channels ensures the generation of action potentials and neuronal polarity. At the AIS, the concentration of voltage-gated ion channels ensures the generation of action potentials (Clark et al., 2009), whereas the peculiar nature of the AIS scaffold maintains the segregation between the axonal and somatodendritic compartment for membrane constituents and for intracellular trafficking (Grubb and Burrone, 2010b). At the molecular level, AIS functions require the segregation of the cytoskeletal adaptor ankyrin G (ankG; Salzer, 2003; Ogawa and Rasband, 2008). The ankG–βIV-spectrin scaffolding complex not only concentrates Nav1 sodium channels (Srinivasan et al., 1988; Zhou et al., 1998; Garrido et al., 2003; Lemaillet et al., 2003; Hedstrom et al., 2007) but also Kv7 potassium channels (Chung et al., 2006; Pan et al., 2006; Rasmussen et al., 2007) and cell adhesion molecules neurofascin-186 (NF-186) and neuronal cell adhesion molecule (NrCAM; Davis et al., 1996; Dzhashiashvili et al., 2007; Hedstrom et al., 2007). Importantly, the interaction between ankG and Nav1 channels is modulated by protein kinase CK2 (CK2) phosphorylation. CK2 concentrates at the AIS, phosphorylates the ankyrin-binding motif of Nav1 channels, and consequently regulates Nav1 interaction.
with ankG (Bréchet et al., 2008). In addition, Kv1 voltage-gated potassium channels are concentrated at the distal AIS in certain types of neurons (Inada et al., 2006; Lorincz and Nusser, 2008).

A handful of studies have revealed that the functional organization of the AIS contributes to the establishment and the maintenance of neuronal polarity. Hedstrom et al. (2008) recently demonstrated that axons acquire the molecular characteristics of dendrites upon AIS disassembly, a result that was also confirmed in vivo (Sobotzik et al., 2009). This contribution occurs through intracellular and cell surface mechanisms, which are still poorly understood. Two studies have shown that the AIS acts as a cytoplasmic filter controlling the entry of vesicles in axons (Burack et al., 2000; Song et al., 2009). At the plasma membrane, the AIS forms a diffusion barrier that maintains the differences in protein and lipid composition between the somatodendritic and the axonal domain of neurons (Kobayashi et al., 1992; Nakada et al., 2003). Importantly, this fence restrains diffusion of axonal proteins independently of their ability to bind to ankG (Winckler et al., 1999; Boiko et al., 2007; Song et al., 2009). However, the mechanisms of the diffusion barrier and its assembly have yet to be determined.

The formation and the maintenance of the AIS depend on ankG expression (Zhou et al., 1998; Hedstrom et al., 2007), and in developing hippocampal neurons, its assembly occurs as soon as the axon starts elongating (stage 2–3 neurons; Boiko et al., 2007; Yang et al., 2007; Bréchet et al., 2008; Martin et al., 2008). Because the segregation of the AIS components appears before the general diffusion barrier (Nakada et al., 2003), this opens the possibility that ankG-dependent tethering is sufficient to restrain the surface diffusion of interacting proteins like Nav1 and adhesion protein. Therefore, the present study aims to analyze the precise role of ankG in the restriction of ion channel diffusion and the potential role of CK2 phosphorylation in this process in an attempt to unravel the mechanisms of the formation of a diffusion barrier at the AIS. We used a construct bearing the ankyrin-binding motif of Nav1.2, whose interaction with ankG is regulated by protein kinase CK2 phosphorylation (Bréchet et al., 2008). We were able to measure the surface diffusion of various Kv-Nav tetrameric ion channel constructs with different ankyrin binding properties. To do so, we used single-particle tracking (SPT; Borgdorff and Choquet, 2002; Tardin et al., 2003) with semiconductor quantum dot (QD) labeling (Dahan et al., 2003). Experiments in N2A cells demonstrated that ankG binding restricts the diffusion of chimeric ion channels and that phosphorylation of the ankyrin-binding motif by CK2 modulates this immobilization. In immature hippocampal neurons, ankG highly restricts the diffusion of ion channels in a CK2-dependent manner, as soon as it concentrates at the AIS. In mature neurons, ankG binding is still necessary for full immobilization, although the appearance of the diffusion barrier also slows the diffusion of proteins that do not bind ankG. Altogether, our findings strongly suggest that the immobilization of Nav1 is controlled by ankG from early stages. Such an immobilization occurs independently of the AIS diffusion barrier and may constitute the initial step toward its establishment.

**Results**

ankG directly restricts ion channel diffusion when expressed in a neuronal cell line

To determine the effect of ankG on the surface diffusion of Nav1 channels, we first chose to heterologously express ankG and an ion channel chimera in neuroblastoma N2A cells. The C terminus of Kv2.1, which dictates its somatodendritic clustering (Lim et al., 2000), was deleted and replaced with the 1,080–1,203

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**Figure 1.** ankG restricts Kv-Nav diffusion at the surface of N2A cells. (A) Schematic representation of Kv-Nav chimera in which the C terminus of Kv2.1 was substituted by a segment encompassing the ankyrin-binding motif of Nav1.2. Note the presence of the tetramerization domain T1 in the N terminus of Kv2.1 and of an extracellular myc tag. (B) Surface expression of Kv-Nav in ankG-GFP-positive cells. Surface Kv-Nav was immunodetected with an antibody to myc (red), and ankG signal corresponds to the GFP fluorescent signal on fixed cells (green). Bar, 10 μm. (C and E) Representative examples of QD trajectories corresponding to Kv-Nav in GFP (red, C) and ankG-GFP–expressing N2A cells (black, E). (D and F) DIDC of QDs stuck on the glass coverslips (gray). Trajectories for GFP (n = 198) and ankG-GFP (n = 253) conditions were analyzed from three and four independent experiments, respectively. (G) Histogram of the mean values ± SEM for the immobile population (percentage) of Kv-Nav in GFP and ankG-GFP conditions. (H) MDC (25–75% IQR) for the mobile population of Kv-Nav in GFP and ankG-GFP conditions. MW: ***, P < 0.001.
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In cells expressing GFP, surface Kv-Nav was highly mobile, and the DIDC exhibited a broadly spread mobile population (Fig. 1, C and D). In ankG-GFP–expressing cells, the Kv-Nav trajectories were visually more restricted than the trajectories recorded in GFP-expressing cells (Fig. 1 E), and Kv-Nav DIDC was characterized by the appearance of a markedly less mobile population (Fig. 1 F). Compared with GFP, ankG-GFP expression strongly increased the population of immobile Kv-Nav, which rose from 6 ± 1 to 34 ± 4% (Fig. 1 G). The MDC of mobile particles significantly decreased between GFP and ankG-GFP conditions (0.025 µm².s⁻¹, interquartile range [IQR] of 0.0085/0.048 µm².s⁻¹, and 0.014 µm².s⁻¹, IQR of 0.0024/0.032 µm².s⁻¹, respectively; Mann and Whitney test [MW], P < 0.0001; Fig. 1 H). These data indicate that ankG restricts Kv-Nav diffusion in N2A cells.

Modulation of ankG binding affects the surface diffusion of ion channels

Neuronal sodium channels bind to ankG through their ankyrin-binding motif, which spans from amino acids 1,102 to 1,128 in the II–III intracellular loop for Nav1.2 (Garrido et al., 2003; Bréchet et al., 2008). We recently showed that this interaction is regulated by CK2 phosphorylation (Bréchet et al., 2008). Nav1.2 and ankG associate with an apparent affinity constant \( (K_d) \) of 1.2 ± 0.4 × 10⁻⁶ and 0.9 ± 0.7 × 10⁻⁹ M before and after

segment of Nav1.2 that encompasses the ankyrin-binding motif (Nav1.2-Nav1.2, further called Kv-Nav; Fig. 1 A; Garrido et al., 2003; Bréchet et al., 2008). The resulting construct can tetramerize (Li et al., 1992), leading to a complex that has a membrane topology similar to Nav channels and bears four ankyrin-binding motifs. In N2A cells, expression of endogenous ankG was not detected by immunostaining (unpublished data). Kv-Nav was uniformly expressed at the plasma membrane in N2A cells (Fig. 1 B) in contrast to what is observed in human embryonic kidney cells in which Kv-Nav and ankG formed aggregates (Garrido et al., 2003), and transfected ankG-GFP displayed a homogenous submembrane localization (Fig. 1 B). This allowed us to analyze the effect of ankG on Kv-Nav mobility using an SPT method to measure surface diffusion (Borgdorff and Choquet, 2002; Tardin et al., 2003). Kv-Nav was coexpressed in N2A cells with either GFP or ankG-GFP, and surface channels were labeled on living cells with QDs using their myc extracellular tag. Rapid time-lapse imaging of individual QDs allowed us to analyze a large number of individual trajectories from which the distribution of the instantaneous diffusion coefficients (DIDC) could be determined. From the DIDC, we chose the maximum diffusion coefficient (D) of immobile QDs lying on the glass coverslip (D ≤ 0.00075 µm².s⁻¹) as the threshold, allowing us to define the immobile population (Fig. 1, D and F). Key mobility parameters such as median instantaneous diffusion coefficients (MDCs) and mean square displacement (MSD) could then be extracted for the mobile particles (see Trajectory analysis in Materials and methods).

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ankG immobilizes ion channels at the nascent AIS of neurons

In the nervous system, the subcellular localization of ankG is highly specific, being segregated at the AIS and in the nodes of Ranvier (Kordeli et al., 1995). ankG accumulation also occurs at the AIS of developing hippocampal neurons maintained in vitro (Garrido et al., 2003; Boiko et al., 2007). In these cells, the AIS molecular assembly has already begun after day in vitro (DIV) 4 (Boiko et al., 2007; Yang et al., 2007; Bréchet et al., 2008; Martin et al., 2008), as visualized by the consistent immunostaining for ankG, Nav1, and NF-186 (Fig. 3 A). The AIS of cultured hippocampal neurons then develops a diffusion barrier that restricts the mobility of membrane proteins and phospholipids, but this general diffusion barrier is not yet formed in DIV 4 hippocampal neurons (Nakada et al., 2003; Boiko et al., 2007).

The mutation of the four serine residues (S1112, S1123, S1124, and S1126) to alanine (4SA mutant) in the Nav1.2 ankyrin binding site not only abolished the CK2-mediated affinity enhancement but also prevented the negative dominant effect of Kv-Nav on Nav accumulation at the AIS. Furthermore, these four serine mutations combined with the mutation of the glutamate residue E1111 (EA4SA mutant) fully abrogated the interaction of the Kv-Nav construct with ankG (Bréchet et al., 2008). Thus, these three structurally identical Kv-Nav constructs differing in their ankyrin-binding motif offer the unique opportunity to study the relationship between the strength of ankyrin binding and the surface mobility of ion channels (Fig. 2 A). It must be noted that these constructs are tetramers that bear four ankyrin-binding motifs, exacerbating their binding to ankG and its modulation in the different mutants. We measured the diffusion of site-directed Kv-Nav mutants in N2A cells expressing either GFP or ankG-GFP. In GFP cells, the immobile population of Kv-Nav, Kv-Nav 4SA, and Kv-Nav EA4SA were 6 ± 1, 7 ± 2, and 4 ± 1%, respectively, with no significant difference between the three constructs (unpublished data). In ankG-GFP–expressing cells, the complete loss of ankG interaction (Kv-Nav EA4SA; Bréchet et al., 2008) induced a large shift of the DIDC toward higher values of diffusion coefficients and a significant difference in cumulative frequencies (Kolmogorov-Smirnov test [KS], P < 0.0001) when compared with Kv-Nav (Fig. 2, B and D). The immobile population decreased from 34 ± 4 to 13 ± 1% (MW, P = 0.0095; Fig. 2 F), and the MDC of the mobile population rose from 0.014 µm2.s−1, IQR of 0.0024/0.032 µm2.s−1, to 0.23 µm2.s−1, IQR of 0.0059/0.066 µm2.s−1, respectively (MW, P < 0.0001; Fig. 2 G). The diffusion behavior of the phosphodeficient Kv-Nav 4SA mutant (Bréchet et al., 2008) was also affected when compared with Kv-Nav (Fig. 2, C and E). The cumulative frequency of diffusion coefficients of Kv-Nav 4SA (Fig. 2 E) was significantly different from that of Kv-Nav and Kv-Nav EA4SA (KS, P < 0.0001 and P = 0.001, respectively) as well as the immobile population (18 ± 1%; MW, P = 0.0286 and P = 0.0190, respectively; Fig. 2 F) and the MDC of the mobile population (0.016 µm2.s−1, IQR of 0.0052/0.042 µm2.s−1; MW, P = 0.0242 and P = 0.0053, respectively; Fig. 2 G). These findings indicate that site-directed mutants with decreasing affinities for ankG exhibited increasing diffusion speeds, highlighting the importance of ankG binding for chimeric ion channel immobilization at the plasma membrane.
We next examined how modulation of ankG binding properties impacts the surface mobility of ion channels in young neurons. Kv-Nav 4SA, which binds ankG with a reduced affinity, still concentrates at the AIS of DIV 4 neurons (Fig. 4 A), similar to what was observed in DIV 10 neurons (Bréchet et al., 2008). However, the diffusion properties of Kv-Nav 4SA were significantly different from those of Kv-Nav, as a shift toward higher diffusion coefficients was seen in the DIDC and in the cumulative frequency (KS, P = 0.002; Fig. 4, B and C). It should be noted that the significant enhancement of mobility observed in the cumulative frequency of diffusion coefficients was not associated with a significant change of the immobile population (77 ± 6% for Kv-Nav and 57 ± 6% for Kv-Nav 4SA; Fig. 4 D) nor in the MDC of the mobile population (0.0015 µm².s⁻¹, IQR of 0.0010/0.011 µm².s⁻¹, for Kv-Nav and 0.0413 µm².s⁻¹, IQR of 0.0018/0.016 µm².s⁻¹, for Kv-Nav 4SA; Fig. 4 E). These results indicate that the mutation of CK2-targeted serines alters Kv-Nav immobilization in young neurons. To further assess the role of CK2 phosphorylation on Kv-Nav immobilization, we analyzed Kv-Nav diffusion upon acute inhibition of CK2 activity using DMAT (2-dimethylamino-4,5,6,7-tetrabromo1H-benzimidazole), a potent CK2 inhibitor (Pagano et al., 2004). Kv-Nav diffusion was first measured in control medium (vehicle condition; t = 0 min; Fig. 5 A). After replacement of the medium with an equivalent solution supplemented with DMAT, the movements of the same QD-labeled ion channels were then repeatedly recorded for a further 10–25 min. The mean diffusion coefficients were calculated for this period (t = 10–25 min; Fig. 5 A) and compared with the initial coefficients. After 10–25 min of DMAT application, the MDC...
of initially immobile QDs increased significantly (from 0.00027 \( \mu m^2 s^{-1} \), IQR of 0.00013/0.00043 \( \mu m^2 s^{-1} \), to 0.00060 \( \mu m^2 s^{-1} \), IQR of 0.00027/0.0029 \( \mu m^2 s^{-1} \); Wilcoxon signed rank test [WSR], \( P = 0.0004 \); Fig. 5 A). This was not observed in control cells treated with vehicle instead of DMAT (from 0.00023 \( \mu m^2 s^{-1} \), IQR of 0.000098/0.00040 \( \mu m^2 s^{-1} \), to 0.00015 \( \mu m^2 s^{-1} \), IQR of 0.000091/0.00056 \( \mu m^2 s^{-1} \); Fig. 5 B). We next examined the effect of DMAT on the diffusion of the phosphodeficient mutant Kv-Nav 4SA. No significant difference was measured in the MDC before and after 10–25 min of either DMA T or vehicle applications (from 0.00020 \( \mu m^2 s^{-1} \), IQR of 0.00010/0.00025 \( \mu m^2 s^{-1} \), to 0.00023 \( \mu m^2 s^{-1} \), IQR of 0.00017/0.00041 \( \mu m^2 s^{-1} \), for DMAT and from 0.00022 \( \mu m^2 s^{-1} \), IQR of 0.000086/0.00047 \( \mu m^2 s^{-1} \), to 0.00031 \( \mu m^2 s^{-1} \), IQR of 0.00024/0.00050 \( \mu m^2 s^{-1} \), for vehicle; Fig. 5, C and D). Comparison across conditions between DMAT and vehicle treatments indicated different MDCs for Kv-Nav, but not for Kv-Nav 4SA (MW for Kv-Nav, \( P = 0.0078 \); Fig. 5, C and D). Altogether, these data strongly suggest that inhibition of CK2 phosphorylation is able to alter Kv-Nav mobility at the AIS.

We next examined whether the increase in mobility induced by CK2 inhibition could later destabilize the concentration of ankyrin-binding ion channels at the AIS. We quantified the immunostaining of ankyrin and endogenous Nav1 channels in neurons treated for 4, 8, or 24 h with either DMAT or vehicle. The inhibition of CK2 activity induced a progressive decrease in Nav1 labeling at the AIS (80 ± 5% of control value after 4 h, 77 ± 7% after 8 h, and 69 ± 6% after 24 h), a process that seems to occur before perturbation of ankyrin concentration (decreasing to 86 ± 55% after 4 h, 86 ± 6% after 8 h, and 48 ± 4% after 24 h; Fig. 5 E). Collectively, these results show that binding affinity, restriction of diffusion, and AIS concentration are correlated in young neurons. They also demonstrate that CK2 can modulate Kv-Nav binding to ankyrin and Kv-Nav immobilization in the nascent AIS.

**ankG restricts ion channel diffusion at the AIS of mature neurons**

We then studied the relationship between ankyrin binding and the restriction of ion channel diffusion in mature neurons, which develop a general diffusion barrier at the AIS (Winckler et al., 1999; Nakada et al., 2003). To discriminate between an effect driven by direct ankyrin binding from the effect of the general diffusion barrier, we compared the diffusion behaviors of Kv-Nav, Kv-Nav 4SA, and Kv-Nav EA4SA in DIV 10 neurons. A loss of compartmentalization was observed for Kv-Nav EA4SA, but not for Kv-Nav 4SA (Fig. 6 A), in agreement with our previous study (Bréchet et al., 2008). In DIV 10 neurons, the diffusion properties of Kv-Nav EA4SA were different when compared with Kv-Nav (Fig. 6 B): the DIDC and the cumulative frequency of diffusion coefficients shifted toward higher values for Kv-Nav EA4SA (KS, \( P < 0.0001 \)), and the percentage of the immobile population dropped to 43 ± 3% (MW, \( P = 0.012 \); Fig. 6 C). Note that the MDC of the Kv-Nav EA4SA mobile population was not significantly different from that of Kv-Nav (0.0075 \( \mu m^2 s^{-1} \), IQR of 0.0018/0.017 \( \mu m^2 s^{-1} \); Fig. 6 D). In comparison, Kv-Nav and Kv-Nav 4SA displayed similar diffusion properties (Fig. 6, B and C); we did not find any significant differences in the DIDC, the cumulative frequency of diffusion coefficients (Fig. 6, B and C), the immobile population (78 ± 4% for Kv-Nav and 72 ± 8% for Kv-Nav 4SA), and the MDC of the mobile population (0.0028 \( \mu m^2 s^{-1} \) for Kv-Nav and 0.0074 \( \mu m^2 s^{-1} \) for Kv-Nav 4SA; IQR of 0.0019/0.021 and 0.0017/0.021 \( \mu m^2 s^{-1} \), respectively; Fig. 6, B and C). These observations suggested that CK2 modulation of Kv-Nav mobility might be occluded in DIV 10 neurons in contrast to what was observed in DIV 4 neurons (Figs. 4 and 5). Therefore, we analyzed Kv-Nav diffusion upon acute inhibition of CK2 activity in a similar manner as described for young neurons (Fig. 5). From 10 to 20 min after drug application, the MDC of initially immobilized channels varied significantly in DMAT but not in vehicle conditions (from 0.00013 \( \mu m^2 s^{-1} \), IQR of 0.000080/0.000022 \( \mu m^2 s^{-1} \), to 0.00020 \( \mu m^2 s^{-1} \), and 0.000012/0.000085 \( \mu m^2 s^{-1} \), for DMAT; WSR, \( P = 0.0186 \); and from 0.00016 \( \mu m^2 s^{-1} \), IQR of 0.000072/0.00039 \( \mu m^2 s^{-1} \), to 0.00026 \( \mu m^2 s^{-1} \), IQR of...
neurons (Fig. 6, F–I). NrCAM-mCherry coexpression was used to visualize the AIS. The percentage of the immobile population of GFP–Kv-Nav 4SA and of GFP–Kv-Nav EA4SA (71 ± 2, 75 ± 2, and 32 ± 6%, respectively; MW, P < 0.0001 for both pairs; Fig. 6 H). The half-recovery time of the three constructs was in the same range of magnitude and did not differ significantly (Fig. 6 I). This independent measurement of Kv-Nav mobility confirms that, in mature neurons, a direct interaction with ankG restricts diffusion of ion channels.

We next conducted experiments using FRAP (Bats et al., 2007) to compare the fluorescence recovery of GFP–Kv-Nav, GFP–Kv-Nav 4SA, and GFP–Kv-Nav EA4SA in DIV 10 neurons (Fig. 6, F–I). NrCAM-mCherry coexpression was used to visualize the AIS. The percentage of the immobile population of GFP–Kv-Nav 4SA and of GFP–Kv-Nav was higher than that of GFP–Kv-Nav EA4SA (71 ± 2, 75 ± 2, and 32 ± 6%, respectively; MW, P < 0.0001 for both pairs; Fig. 6 H). The half-recovery time of the three constructs was in the same range of magnitude and did not differ significantly (Fig. 6 I). This independent measurement of Kv-Nav mobility confirms that, in mature neurons, a direct interaction with ankG restricts diffusion of ion channels.
an AIS marker (Fig. S2). In this condition, Kv-Nav EA4SA diffusion properties were similar for proximal dendrites and for the AIS, similar to what was observed in the presence of NrCAM-GFP (Fig. 7, A–C). These data show that an ion channel deficient for ankG binding exhibited a restricted mobility at the AIS of mature neurons, but not of immature neurons. This is likely to result from the appearance of a general diffusion barrier, in line with previous studies (Winckler et al., 1999; Nakada et al., 2003). Altogether, these findings revealed that a two-step mechanism of diffusion restriction takes place at the AIS.

As soon as the scaffold protein concentrates at the AIS, before the assembly of the diffusion barrier (DIV 4), a tight and CK2-regulated binding to ankG is required to immobilize Nav channels. Once the barrier is formed (DIV 10), ion channel immobilization still requires ankG binding, but the presence of the barrier also immobilizes proteins that bind weakly or do not bind ankG.

Discussion

In the present study, we show that ankG restricts diffusion of Kv-Nav chimeric ion channels through interaction with the Nav1.2 ankyrin-binding motif in N2A cells and hippocampal neurons. In young neurons, CK2 is able to modulate the immobilization of Kv-Nav. In mature neurons, ankG is still necessary for Kv-Nav immobilization, and CK2 modulation is attenuated. Our findings demonstrate that a CK2-regulated binding to ankyrin is initially required to immobilize Nav channels at the AIS before the assembly of the diffusion barrier. Once formed, the diffusion barrier can partially supplement the role of ankG in immobilizing ion channels, as demonstrated with Kv-Nav mutants.
with reduced ankyrin binding. Thus, early immobilization of ankyrin-binding proteins may constitute a preliminary step in the formation of the diffusion barrier (Winckler et al., 1999; Nakada et al., 2003).

**Diffusion of ankyrin-binding proteins at the AIS**

SPT with QDs was first used to study diffusion of the glycine receptor in spinal cord neurons (Dahan et al., 2003). The main benefit of this technique is that it allows the investigator to obtain a detailed DIDC rather than a mean coefficient of diffusion-like ensemble methods such as FRAP (Triller and Choquet, 2008). The diffusion coefficient values obtained for Kv-Nav constructs are in the order of $D = \sim 0.001 \, \mu m^2 \cdot s^{-1}$ in both young and mature neurons. Compared with other membrane proteins, this places AIS channels at the slowest part of the diffusion spectrum (for review see Groc and Choquet, 2008). Early results suggested a slow diffusion of Nav1 channels at the axon hillock ($D = \sim 0.015 \, \mu m^2 \cdot s^{-1}$; Angelides et al., 1988), later confirmed by SPT of toxin-tagged channels ($D = \sim 0.01 \, \mu m^2 \cdot s^{-1}$; Nakada et al., 2003). For adhesion proteins, a diminished diffusive properties of endogenous Nav channels that bear a single ankyrin-binding motif.

The tetravalence of Kv-Nav complexes is likely caused by the higher sensitivity of the SPT/QD method. Alternatively, immobilization could be enhanced by the tetrameric nature of Kv-Nav constructs that bear four ankyrin-binding motifs. The tetravalence of Kv-Nav complexes is likely to exacerbate the differences in the ankyrin binding properties of Kv-Nav and its mutants and could have helped to discriminate their differences in diffusive properties. Nevertheless, one of the main goals of future studies will be to measure the diffusive properties of endogenous Nav channels that bear a single ankyrin-binding motif.

**Diffusion-trapping model for accumulation of ankyrin-binding proteins at the AIS**

Studies of chimeras containing different portions of the Nav1.2 sodium channel have shown that these constructs are sent to both somatodendritic and axonal compartments before being selectively endocytosed (Fache et al., 2004) and retained at the AIS because of their interaction with ankG via the ankyrin-binding motif (Garrido et al., 2003; Lemaitre et al., 2003). By using N2A cells and neurons at different stages of development, we now show that the slow diffusion of Kv-Nav depends on its binding to ankyrin. Downstream of targeting mechanisms, this supports a diffusion-trapping mechanism for ankyrin-binding channels at the AIS. Trapping of ion channels and adhesion proteins by ankG could explain how they progressively concentrate at the AIS in developing neurons (Xu and Shrager, 2005; Boiko et al., 2007; Hedstrom et al., 2007). Interestingly, the ankG–Nav1 interaction is regulated by CK2 phosphorylation, and the restriction of CK2 to the AIS can explain why Nav1 channels are preferentially trapped by ankG at the AIS rather than by ankB further down the axon (Bréchet et al., 2008). Ankyrin-based trapping of L1CAM by ankB has been demonstrated in axonal growth cones (Gil et al., 2003) and is also dependent on phosphorylation by an unknown kinase (Garver et al., 1997). Here, perturbing ankG–Nav1 interactions by inhibiting CK2 with the DMAT inhibitor affects ion channel diffusion and accumulation at the AIS. CK2 inhibition leads to an enhanced diffusion of Kv-Nav after 10 min and to a decrease in endogenous Nav1 concentration at the AIS after 4–8 h. The difference between the short-term effect on diffusion and the long-term effect on localization could seem paradoxical. However, differences in diffusion can be very slow to affect steady-state localization of membrane proteins, as shown by mathematical modeling of neurotransmitter receptors at the postsynaptic density (Holcman and Triller, 2006; Sekimoto and Triller, 2009).

**Primary role of ankG in restricting diffusion at the AIS**

The primary role of ankG in AIS assembly and maintenance is now established (Zhou et al., 1998; Hedstrom et al., 2007, 2008), but its role in the diffusion barrier has not been clearly addressed yet (Lasiecka et al., 2008). Previous studies have shown that there is a correlation between ankyrin concentration at the AIS and the restriction of the diffusion of neurofascin (Boiko et al., 2007) and lipids in older neurons (Nakada et al., 2003). In a recent study, knocking down ankG expression allowed VAMP2 to diffuse more freely at the AIS of young neurons (Song et al., 2009). First, ankG is able to specifically restrict the diffusion of ankG-binding constructs before the diffusion barrier appears. Second, after the diffusion barrier has established at DIV 10, all constructs are slowed at the AIS compared with proximal dendrites, but constructs that bind to ankG are more immobile than constructs that do not. We could separate the immobilization of ankyrin-binding proteins from the general immobilization of membrane proteins caused by the diffusion barrier because, in our system, we observed a concentration of ankG at the AIS by DIV 4, similar to other studies (Hedstrom et al., 2007; Song et al., 2009), before seeing an effect of the diffusion barrier that is present at DIV 10. An interesting question, given the large spread of DIDC for Kv-Nav, is the relative contribution of ankG mobility, as scaffolding complexes have been shown to be dynamic (Specht and Triller, 2008). However, the mobility of such scaffolds is usually very low compared with the diffusion of membrane proteins. The DIDC spread is more likely caused by rapid association–dissociation events with ankG along the trajectories, resulting in a diminished diffusion coefficient.

**Subsequent steps in the diffusion barrier assembly**

Our results show that ankyrin-binding proteins are immobilized at the AIS as soon as DIV 4, whereas the mobility of ankyrin-noninteracting proteins was restricted at a later stage of development. This suggests that immobilization of ankyrin-binding proteins is a preliminary step in the assembly of the diffusion barrier. In the “fence and pickets” model of the AIS diffusion barrier (Nakada et al., 2003), ankG acts as a fence post to recruit ankyrin-binding membrane proteins as pickets. If ankyrin-binding pickets are sufficient to slow down all AIS membrane proteins, overexpressing NrCAM-GFP pickets at the AIS of...
DIV 4 neurons should have a significant effect on the diffusion of Kv-Nav E4SA, which does not interact with ankG. However, we observed that NrCAM-GFP only slightly decreased Kv-Nav E4SA diffusion (Fig. 6). This could be because overexpression of NrCAM-GFP is not sufficient to reach a critical concentration of pickets (Nakada et al., 2003) or NrCAM-GFP is not as functional as endogenous NrCAM, as it lacks the extracellular FnIII domain that was swapped for GFP (Falk et al., 2004). More likely, the presence of more membrane ankyrin-binding proteins is not sufficient to create a diffusion barrier. The AIS still develops and matures between DIV 4 and DIV 10, with the assembly of additional components. βIV-Spectrin (Berghs et al., 2000) is likely to be another constituent of the fence, although its depletion affects the diffusion of only L1CAM along the AIS (Nishimura et al., 2007). Specific lipids that are organized and stabilized by membrane proteins, such as cholesterol, which interacts with NrCAM through its palmitoylated residues (Ren and Bennett, 1998), could also contribute to the restriction of diffusion. Another probable factor is the appearance of a specific brevican-containing extracellular matrix that ensheathes the AIS in mature neurons (Hedstrom et al., 2007) and is likely to affect diffusion of membrane proteins (Frischknuecht et al., 2009).

Physiological role of the modulation of protein mobility at the AIS

This major role of a scaffolding protein in restricting membrane protein diffusion and ultimately localization is reminiscent of what has been consistently found at the postsynaptic compartment. Direct or indirect interactions of neurotransmitter receptors with scaffolds drive receptor concentration and play a role in modulating their presence at the synapse. At excitatory synapses, AMPA receptors associate with stargazin, resulting in the immobilization of AMPA–stargazin complexes (Bats et al., 2007). At inhibitory synapses, γ-aminobutyric acid and glycine receptors bind to gephyrin, which leads to their concentration at the postsynaptic density (Renner et al., 2008). Furthermore, modulation of receptor concentration at synapses by neuronal activity could occur through regulation of the scaffold–receptor binding strength, suggesting a mechanism for synaptic plasticity (Ehrensperger et al., 2007; Newpher and Ehlers, 2008). Here, we clearly show that Kv-Nav diffusion at the AIS is linked to its capability to bind ankG, demonstrating that a regulation of scaffold–membrane protein interaction can drive differences in diffusional behavior. It must be noted that such a regulation implies that the scaffold is in excess relative to the membrane protein, which seems to be the case for ankG at the AIS (Davis and Bennett, 1984). In addition, the modulation of ankG binding to Kv-Nav is caused by phosphorylation by a specific kinase (CK2; Bréchet et al., 2008), and we were able to detect the regulatory role of CK2 on Kv-Nav diffusion in young neurons. As the AIS has recently been shown to be modulated by neuronal activity (Grubb and Burrone, 2010a; Kuba et al., 2010), it would be very interesting to test whether variation in Nav1 diffusive properties, as well as its dynamic regulation by CK2 phosphorylation, play a role in this new mechanism of neuronal intrinsic plasticity.
Inhibition of CK2 activity

Inhibition of CK2 activity was performed using the CK2 inhibitor DMAT (Pagano et al., 2004). DMAT (EMD) was dissolved in DMSO at 10 mM. For long-term treatment experiments, DMAT was added to the culture medium at a final concentration of 50 µM. Corresponding amounts of DMSO (vehicle) were added to the medium of control cells.

For DMAT acute application experiments, coverslips were first mounted in an open chamber (Life Imaging Services) with imaging buffer, transfected neurons were chosen, and control QD trajectories were recorded. Then, the imaging buffer was replaced by the same medium supplemented with 50 µM DMAT or DMSO as a vehicle, and the same QDs were then imaged every 5 min for 20–25 min. Imaging was performed as described in the previous section with the exception that GFP and QDs were detected using different excitation and emission filters (bp 470/40–bp 525/50 for GFP signal and bp 525/45–bp 605/20 for QD signal).

FRAP experiments

Experiments were conducted on a wide-field FRAP imaging system (LSI; Institut Curie/Roper Industries) run by MetaMorph software using routines developed at the imaging platform of the Curie Institute. The microscope (DM6000; Leica) was equipped with a 63× objective, a monochromator for wide-field fluorescence excitation (Cairn Research), and an emission filter wheel (Sutter Instrument Co.).

Hippocampal neurons were cotransfected with NrCAM-mCherry and GFP-Kv-Nav or mutated GFP-Kv-Nav. The AIS of transfected neurons was visualized by NrCAM-mCherry fluorescence concentration. Conversely, proximal neurites not exhibiting NrCAM-mCherry concentration were considered as proximal dendrites. For FRAP experiments, only the GFP channel was recorded. Cells were mounted in a custom sealed recording chamber in imaging medium (1.45 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM glucose, 10 mM Hepes, 0.2% BSA, pH 7.39, and 280 mOsm) and maintained at 37°C. One to three circular regions (Gaussian profile beam of 1-µm width) on each AIS were bleached by high-intensity illumination using a 100-mW solid-state laser (Coherent, Inc.) for 15 ms at 15 mW, reducing local fluorescence by 50–80%. Recovery was monitored by time-lapse acquisitions (one frame every second for 180 s) with a background-corrected CCD (Cascade II; Photometrics). Illumination intensity was set to prevent observational photobleaching (<10% in 200 frames). Fluorescence intensities were preliminarily corrected for background signal. Fluorescence signal was then normalized and corrected for ongoing photobleaching (<10% in 200 frames).

Confocal microscopy and quantification

Cells were imaged using a confocal microscope (TCS-SP2; LCS or LAS-AF software, Leica). Confocal images were acquired with 40×/1.25 NA and 63×/1.40 NA oil objectives (Leica) at ambient temperature.

Fluorescence was collected as Z stacks with sequential wavelength acquisition. Quantification was performed using Imagel software (National Institutes of Health). Regions of interest corresponding to AIS were manually selected on anG and/or Nav1 images and reported on other channels for intensity measurements. All intensities were corrected for background labeling. For illustration, image editing was performed using ImageJ or Photoshop CS4 (Adobe) and was limited to rolling-ball background subtraction, linear-contrast enhancement, and ISO adjustment.

Statistical analysis

Statistical analyses were performed with Prism software (GraphPad Software, Inc.). The parametric two-tailed t test was used for steady-state localization experiments, and the nonparametric tests (MW, WSR, and KS) were applied for diffusion experiments given to the anomalous nature of data distributions. Comparison of DIDC was performed using a KS.

Online supplemental material

Fig. S1 demonstrates that accumulation of endogenous ankG is sufficient to account for the reduction of Kv-Nav mobility in young neurons. Fig. S2 shows that overexpression of ankG is not sufficient to induce a diffusion barrier in young neurons.

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