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CK2 accumulation at the axon initial segment depends on sodium channel Nav1

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ABSTRACT

Accumulation of voltage-gated sodium channel Nav1 at the axon initial segment (AIS), results from a direct interaction with ankyrin G. This interaction is regulated in vitro by the protein kinase CK2, which is also highly enriched at the AIS. Here, using phosphospecific antibodies and inhibition/depletion approaches, we showed that Nav1 channels are phosphorylated in vivo in their ankyrin-binding motif. Moreover, we observed that CK2 accumulation at the AIS depends on expression of Nav1 channels, with which CK2 forms tight complexes. Thus, the CK2–Nav1 interaction is likely to initiate an important regulatory mechanism to finely control Nav1 phosphorylation and, consequently, neuronal excitability.

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1. Introduction

In mammalian neurons, the generation and propagation of action potentials result from the presence of dense clusters of voltage-gated sodium channels (Nav1) at the axonal initial segment (AIS) and nodes of Ranvier [1,2]. In these two structures, the assembly of specific supra-molecular complexes, composed of numerous partners, such as cytoskeletal scaffold proteins and protein kinases, ensures the high concentration of Nav1 channels [3,4]. One key feature regulating Nav1 channel clustering is their binding to the scaffolding protein ankyrin G (ankG) *via* a highly conserved amino acid motif (ankyrin-binding motif) in their intracellular loop II–III [5,6]. This interaction is regulated *via* CK2-mediated phosphorylation of the ankyrin-binding motif (ABM), which increases the affinity of channels for ankG [7]. Interestingly, CK2 is found highly enriched at the AIS and nodes in vivo, where it most likely phosphorylates the ABM of Nav1 channels. Further, its pharmacological inhibition induces a loss of both Nav1 and ankG accumulation at the AIS [7]. Altogether these observations suggest that CK2-mediated phosphorylation is implicated in in vivo Nav1 clustering. But, to date, no evidence of the ABM being phosphorylated by CK2 in vivo has been shown, underestimating the importance of CK2-mediated phosphorylation in Nav1 clustering.

CK2 is a ubiquitous serine/threonine kinase, which is highly active in brain, especially in cortex and hippocampus [8]. CK2, considered to be constitutively active, exists as a tetramer composed of two catalytic subunits (alpha or alpha prime) and two regulatory beta subunits [9]. Given the participation of CK2 in a myriad of cellular events it is apparent that a number of discrete and independent regulations exist within cells to selectively modulate CK2 activity. Some of these in vivo regulations include control of CK2 expression level, assembly, stability and phosphorylation of either alpha or beta CK2 subunits [8]. Another reported mode of modulating CK2 phosphorylation is the targeting of the kinase to specific structures where it selectively phosphorylates individual substrates [8]. Thus, raising the question on how CK2 is targeted and anchored at the AIS and nodes where it possibly phosphorylates Nav1 and others targets.

In this study, we provide evidence that the ABM of Nav1 channels is phosphorylated in vivo and that Nav1 expression is necessary for CK2 clustering at the AIS. This suggests that CK2-mediated phosphorylation participates in Nav1 clustering in vivo and that its specific localization at the AIS is dependent on Nav1 expression.

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2. Materials and methods

2.1. DNA constructs

Plasmids encoding GST-CK2α, GST-Nav1.2 1080–1203, GFP-Nav1.2 984–1203, myc-Kv2.1-Nav1.2 1080–1203 (Kv2.1-Nav1.2 1080–1203) and its point mutants (Kv2.1-Nav1.2 4SA) were previously described [5,7,10]. DNA sequence containing sequence for shRNA, a loop region of 9 nucleotides (TTCAAGAGA), and flanked by H1 and U6 promoter was chemically synthetized and inserted into pCR^{R}2.1-TOPO (Eurofins mwg/operon). The resulting shRNA expression cassette was then subcloned into EcoR1/BstBI of the modified pFUGW vector [11] where the EGFP was replaced by farnesylated EGFP (from pEGFP-F, Clontech). The resulting pFUfGW-sh CK2 and pFUfGW-sh Nav1 plasmids were verified by DNA sequencing (Beckman Coulter Genomics, UK). The target sequence against rodent CK2α is GAGTTTACACAGATGTTAA. shRNA sequence against Nav1 was previously described [12].

2.2. Generation of the phosphospecific Ab Nav1112P

A synthetic peptide phosphorylated at S1112 (amino acids 1104–1118 of Nav1.2, C-TVPIALGE[pS]DFENLN) and the non-phosphorylated equivalent peptide were synthesized (AGRO-BIO). The phosphopeptide was conjugated to keyhole limpet hemocyanin (EMD) using sulfo-maleimidobenzoyl-NHS ester (Thermo Fisher Scientific), and injected into rabbits for the production of polyclonal antisera. For affinity purification, the phosphorylated and non-phosphorylated peptides were conjugated to SulfoLink coupling resin (Thermo Fisher Scientific) via synthetic N-terminal cysteine residues, and phosphospecific Abs (Nav1112P) were affinity purified by a two-step affinity purification procedure [13]. Phosphospecificity was verified by western blot using recombinant protein GST-Nav1.2 1080–1203 wild type and mutants (S1112A or S1126A) previously phosphorylated and non-phosphorylated. The non-phosphospecific Abs obtained after the purification were used as pan-Nav1 Abs (pan-Nav). Unfortunately, our phosphospecific Nav1112P antibodies were not suitable for immuno-cyto or histo-staining experiments. It is likely that in these conditions the ability of the Abs to recognize target epitopes was affected by the fixation or that the Abs could not access the target epitope as Nav1 ABM was engaged into the interaction with ankG.

2.3. Animals

The use of Wistar rats followed the guidelines established by the European Animal Care and Use Committee (86/609/CEE) and was approved by the local ethics committee (agreement C13-055-8).

2.4. Cell culture, preparation of brain membrane fractions

Rat hippocampal neurons were cultured as described [5], transfected at 7 div using Lipofectamine 2000 (Invitrogen), or nucleofected before plating using Amaxa rat neuron nucleofector kit (Lonza) according to the manufacturer's instructions and fixed at the indicated time points.

Rat hippocampal membranes were prepared at different postnatal days, by homogenization of dissected hippocampi in 0.32 M sucrose, 5 mM sodium phosphate, pH 7.4, 0.1 M NaF, 1 mM EDTA, anti-protease tablet (Roche) as described previously [14]. The pellet of the crude membranes was suspended in the homogenization buffer and protein concentration was determined using the BCA (bicinchoninic acid protein assay) method (Thermo Fisher Scientific). COS-1 cells cultured as described [5], were transiently transfected using the Lipofectamine 2000 (Invitrogen) reagents using the manufacturer's protocols.

2.5. In vitro phosphorylation assay

Purified GST-Nav1.2 1080-1203 and the corresponding mutants S1112A and S1126A (1–5 μg) were suspended in 50 μl of kinase buffer (20 mM Tris–HCl, 50 mM KCl, and 10 mM MgCl2, pH 7.5) containing 2 mM ATP (Invitrogen) then incubated with 10 units of CK2 (New England Biolabs, Inc.) for 30 min at 30 °C. The reaction was stopped by adding reducing sample buffer and samples were analyzed on 12% SDS–PAGE.

2.6. Immunoblotting, GST pull-downs, and alkaline phosphatase treatment

Procedures for immunoblot analysis were performed as reported previously [13]. Rat hippocampus membrane and transfected cell extracts were separated by SDS–PAGE and transferred to nitrocellulose membranes. The membranes were then sequentially incubated with antibodies after normalization to β-tubulin. For GST pull-down assays, COS-1 cells transfected with GFP-Nav1.2 984–1203 or GFP were lysed in 10 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholate, 0.1% BSA, and a complete protease inhibitor tablet (Roche) for 30 min at 4 °C and then were centrifuged at 14,000×g for 15 min at 4 °C. The supernatant was incubated (4 h at 4 °C) with GST-CK2, or GST both previously coupled to glutathione Sepharose 4B following the manufacturer's instructions (GE Healthcare). The beads were then washed with lysis buffer and eluted with reducing sample buffer. The pull-down samples were resolved by SDS/PAGE and were immunoprobed using an anti-GFP antibody. For alkaline phosphatase (AP) treatment, membrane preparations and cell lysates were incubated with or without 100 U/ml of AP (Roche) as reported previously [15].

2.7. Immunofluorescence and antibodies

Neurons were fixed 10 min with 4% paraformaldehyde, then blocked for non-specific binding using 30-min incubation in ICC buffer (0.22% gelatin, 0.066% saponin in phosphate buffer). Cells were then incubated at room temperature (RT) for 1 h with ICC-diluted primary antibodies, rinsed and incubated with ICC-diluted secondary antibodies for 45 min. Coverslips were mounted in FluorSave reagent (Calbiochem). Antibodies used were as follows: anti-microtubule-associated protein 2 (2) chicken polyclonal antibody (Abcam), anti-GFP rabbit polyclonal antibody (ab290, Abcam), anti-ankG mouse monoclonal antibody (N106/65 NeuroMab), anti-Nav mouse monoclonal (K58/35, Sigma–Aldrich), goat polyclonal antibody to CK2α (Santa Cruz Biotechnology, Inc.). Secondary antibodies conjugated to Alexa Fluor 488 and 555 were from Invitrogen; secondary antibodies conjugated to Cy5 and 647 were from Jackson Immunoresearch; and secondary antibodies conjugated to DyLight 405 were from Rockland.

2.8. Image processing and quantification

Immunofluorescence slides were imaged using a confocal microscope (TSC-SP2, Leica or LSM 780, Zeiss). Confocal images were acquired with 63×/1.40 NA oil objectives (Leica and Zeiss) at RT. Fluorescence was collected as z stacks with sequential wavelength acquisition. Quantification was performed using ImageJ software (http://rsb.info.nih.gov/ij/). Image stacks were converted into single maximum intensity *z*-axis projections. For neurons expressing channel chimera regions of interest corresponding to AIS were manually selected on ankG images and reported on other

channels for intensity measurements. Measurements of intensities along the AIS for neurons expressing shRNA were made using NeuronJ software. The AIS intensity ratio was defined as the mean intensity of nucleofected neuron AIS divided by the average of the mean intensities of the AIS of all surrounding non-transfected neurons. Image editing was performed using ImageJ or Photoshop CS5 (Adobe) and was limited to rolling-ball background subtraction, linear contrast enhancement, and gamma adjustment.

3. Results

3.1. Phosphorylation of Nav1 ankyrin-binding motif occurs in vivo

We have previously shown that the interaction between central nervous system-expressed Nav1 channels and ankG is facilitated by CK2-mediated phosphorylation of 4 conserved serines (S1112, S1123, S1124 and S1126 on Nav1.2) of the ankyrin-binding motif (ABM) [7]. But to date, no evidence of any of these serines being phosphorylated in vivo has been provided. To determine if these CK2-regulated serines are phosphorylated in vivo, we generated a rabbit polyclonal Ab against the synthetic peptide TVPIALGEpSDFENLN where the serine 1112 (Nav1.2 sequence) was phosphorylated (Fig. 1A). Phosphospecific antibodies (Nav1112P) were first purified using a two-step immunoaffinity purification procedure involving phosphopeptide enrichment and dephosphopeptide depletion, and then validated by immunoblot analyses of the bacterially expressed GST-Nav1.2 1080–1203 and GST-Nav1.2 1080–1203 phosphorylation-site mutants (S1112A and S1126A) (Fig. 1B). On immunoblot Nav1112P recognized the WT and S1126A samples phosphorylated by the kinase CK2, but none of the S1112A nor the non-phosphorylated WT and S1126A (Fig. 1B). To further validate Nav1112P, we immunoblotted extracts of COS-1 cells expressing the ion channel chimera Kv2.1-Nav1.2 1080–1203, either treated or not by alkaline phosphatase (AP) (Fig. 1C). Nav1112P immunoreactivity was strongly detected in the untreated samples (e.g. phosphorylated Kv2.1-Nav1.2 1080–1203), whereas no bands were visible in the AP-dephosphorylated samples (Fig. 1C, left panel). All together, these results indicate that Nav1112P is specific to the CK2-regulated serine 1112 only when this serine is phosphorylated. We next analyzed whether Nav1112P could detect in vivo phosphorylation of Nav1 channels. Adult rat brain extracts were either treated or not with AP and then immunoblotted with Nav1112P. As shown in Fig. 1C (right panel), Nav1112P strongly recognized Nav1 channels in control rat brain membrane, but not in AP-treated samples, confirming that Nav1 are phosphorylated in their ABM in vivo. We then examined the appearance of this phosphorylation during neuronal development. Dissected rat hippocampus from different post-natal ages, were homogenated and immunolabelled sequentially with Nav1112P, Nav1.2, Nav1.6, pan-Nav1 and finally tubulin Abs (Fig. 1D). As expected during development, Nav1.2 was expressed first in early ages and Nav1.6 appeared in more mature neurons after the first post-natal week [16]. The phosphorylation was present in all ages. But, the comparison of the intensities of both phosphorylated (Nav1112P) and total (Pan-Nav) populations showed that the phosphorylation of the Nav1 occurred more during the first post-natal week. All these results show for the first time that phosphorylation of ABM occurs in vivo during development as well as in adult brain.

3.2. Nav1 expression is necessary for CK2 localization at the AIS

We next asked how CK2 was concentrated at the AIS. Our first hypothesis was Nav1 channels might be a key regulator of CK2 localization at the AIS. To test this, we used a shRNA method to silence Nav1 expression in hippocampal neurons in culture. Freshly dissociated hippocampal neurons were nucleofected with either Nav1 shRNA or control shRNA and then plated. Seven days later, we measured the intensities of Nav1, CK2 and ankG at the AIS of Nav1 shRNA neurons and normalized them with the intensi-

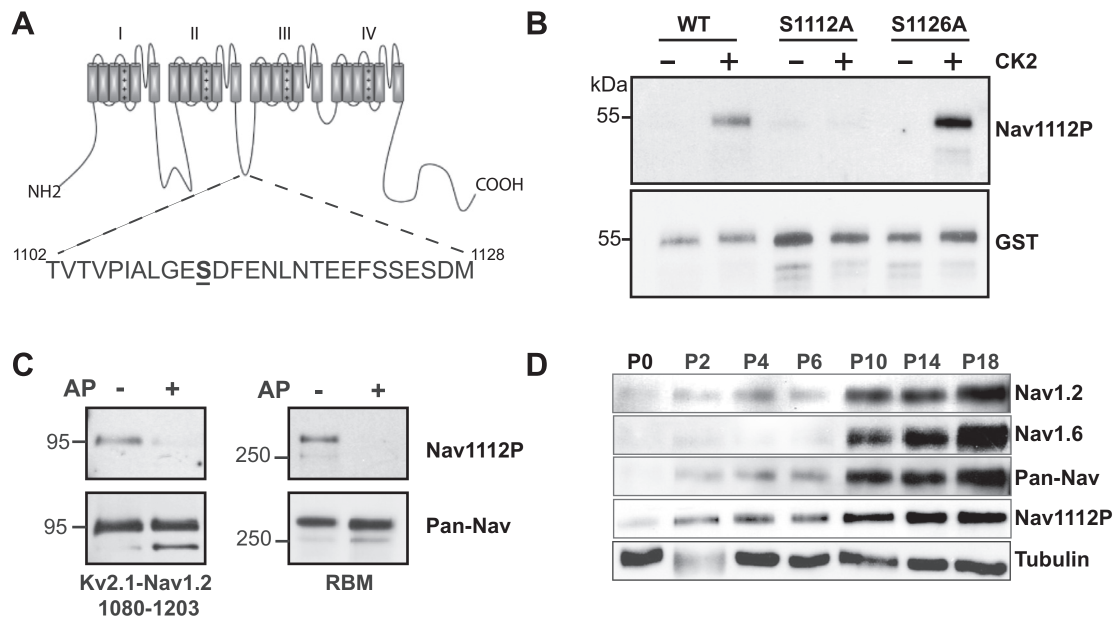

Fig. 1. Ankyrin-binding motif of Nav1 channels is phosphorylated in vivo at the serine 1112. (A) Schematic representation of ABM of Nav1 channels expressed in the CNS. The underlined serine residue indicates the serine 1112 of Nav1.2 against which the phosphospecific antibody was made. (B) GST-Nav1.2 1080-1203 (WT) and phospho-site mutants (S1112A and S1126A) were phosphorylated by recombinant CK2. Samples were analyzed by SDS–PAGE, transferred to a nitrocellulose membrane and revealed by the phosphospecifics antibodies (Nav1112P) and anti-GST antibodies. (C) COS-1 lysates expressing the chimera Kv2.1-Nav1.2 1080-1203 (left panel), and adult rat brain membrane (RBM) (right panel) were dephosphorylated by recombinant alkaline phosphatase (AP). Control and AP-treated samples were analyzed by SDS–PAGE, transferred to nitrocellulose membranes and revealed by the phosphospecifics antibodies (Nav1112P) and rabbit pan-Nav1 antibodies (pan-Nav). (D) Rat hippocampus membrane were prepared at different post-natal days (P), separated by SDS–PAGE and transferred to a nitrocellulose membrane. The membrane was then sequentially incubated with the listed antibodies.

ties found at the AIS of control shRNA neurons (Fig. 2A and B). Neurons expressing Nav1 shRNA, showed ∼60% reduction of Nav1 at their AIS (Fig. 2B, $n = 33$), when compared to control neurons. As shown in Fig. 2A by the asterisk, a significant decrease of CK2 staining at the AIS of nucleofected neurons was seen (∼40% reduction) (Fig. 2B, $n = 26$). A similar reduction in ankG staining at the AIS of nucleofected neurons was also observed (data not shown). To test if silencing of CK2 could reciprocally affect Nav1 AIS localization, we nucleofected hippocampal neurons with CK2 shRNA. Seven days later, we measured ∼25% reduction of CK2 at the AIS of nucleofected neurons compared to control (Fig. 2C, $n = 22$). Similarly, Nav1 expression was reduced at the AIS of neurons expressing CK2 shRNA (Fig. 2C, $n = 28$). Together, these results show that Nav1 is important for CK2 localization at the AIS. They also show that CK2-dependent phosphorylation contributes to Nav1 clustering at the AIS.

3.3. CK2-Nav1 molecular complex modulates CK2-clustering at the AIS

It has been previously shown that CK2 localization and specific activity might be regulated by the formation of tight molecular complexes with its substrates [8]. To determine if CK2 and Nav1 could form such complexes, we performed GST pull-down assays using bacterially expressed GST-CK2 α and GFP-Nav1.2 984–1203 expressed in COS-1 cells. As shown Fig. 3A, GST-CK2 α specifically brought down GFP-Nav1.2 984–1203, suggesting that CK2 directly binds to Nav1 in a stronger fashion than the usual enzyme-substrate brief binding. To test if these CK2-Nav1 complexes were present in neurons, we performed a Nav1-localization displacement assay in hippocampal neurons in culture, using the chimera Kv2.1-Nav1.2 1080–1203 (Fig. 3B). When expressed in 7 DIV neurons, Kv2.1-Nav1.2 1080–1203 displaced endogenous Nav1 from the AIS. Nav1 intensity staining was reduced by ∼60%, while ankG concentration was not changed (Fig. 3C). This was also associated with a loss of CK2 AIS-localization (Fig. 3D) and a robust reduction of CK2-AIS staining of ∼35% was observed. It is important to note that the chimera Kv2.1-Nav1.2 1080-1203 does not contain the entire Nav1.2 II-III loop as GFP-Nav1.2 984–1203 does. To verify that the decrease of CK2-localization at the AIS was a direct consequence of the displacement of endogenous Nav1, we transfected the neurons with the phospho-deficient chimera Kv2.1-Nav1.2 4SA in which the four conserved serines of Nav1.2 ABM were

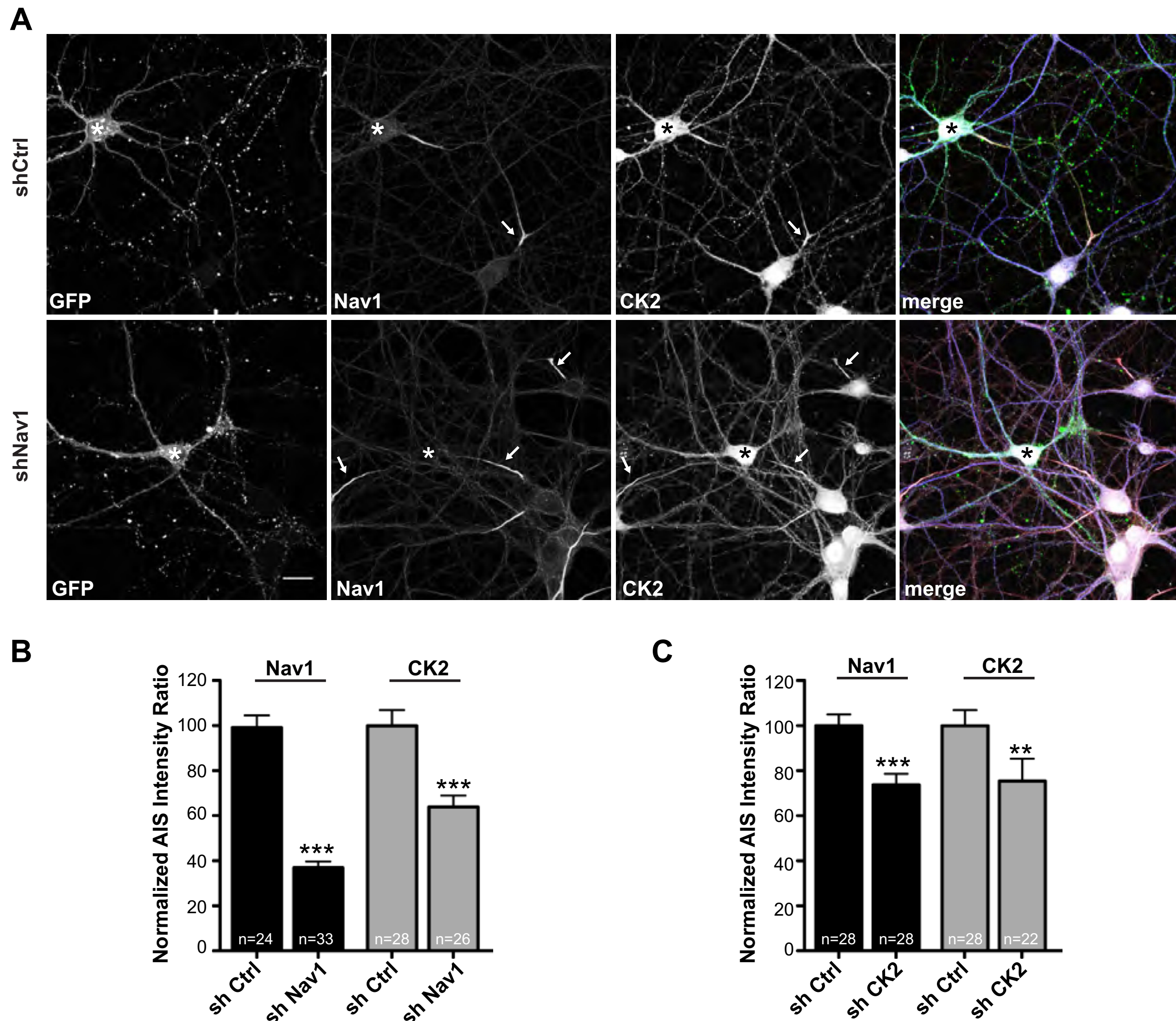

Fig. 2. CK2 localization at the AIS is dependent of Nav1 expression. (A) Hippocampal neurons were nucleofected before plating with control shRNA (shCtrl) or Nav1 shRNA (shNav1) and immunostained at 7DIV for GFP, CK2α and map2. Stars show nucleofected neurons and arrows indicate examples of AIS of non-nucleofected neurons. Scale bar = 20 μm. (B) Effect of Nav1 inhibition on Nav1, CK2α and ankG. AIS localization was determined by quantifying the AIS intensity ratios of neurons expressing shCtrl or shNav1 ($n = 24–33$ neurons from two independent experiments). Bars indicate mean ± SEM. One-way ANOVA post-test comparison, $^{***}P < 0.001$. (C) The effect of CK2α inhibition on Nav1, CK2 α and ankG. AIS localization was determined by quantifying the AIS intensity ratios of neurons expressing shCtrl or CK2α shRNA (shCK2) ($n = 22–28$ neurons from two independent experiments). Bars indicate mean ± SEM. One-way ANOVA post-test comparison, $^{**}P < 0.01$, $^{***}P < 0.001$.

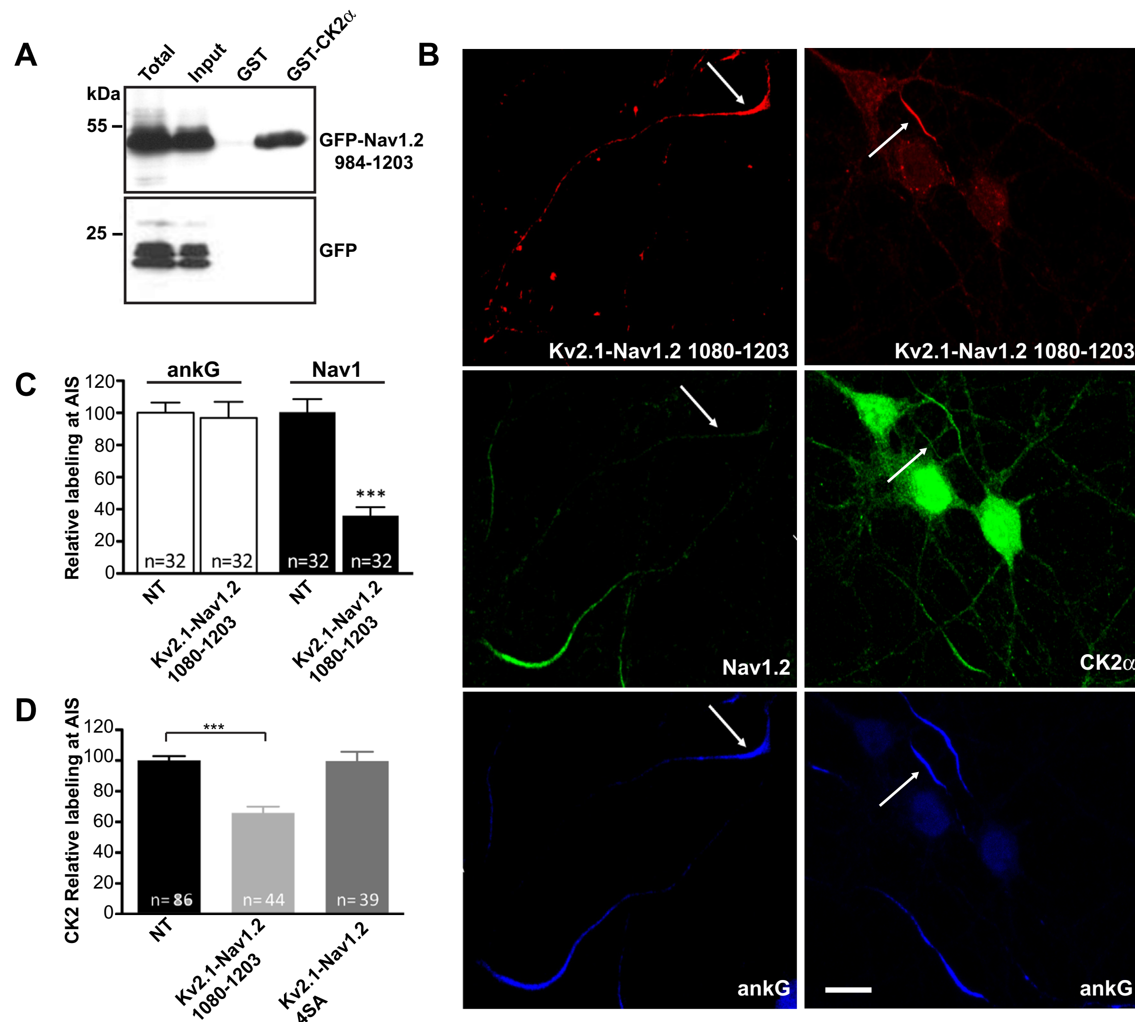

Fig. 3. CK2 forms molecular complex with Nav1. (A) GST pull-down assays. Total (total homogenate), Input (lysate) and products of GST pull-down reactions performed with GST-CK2α on lysate from COS-1 cells expressing GFP-Nav1.2 984-1203 or GFP. GST and GFP were used as a negative control. The gel was immunoblotted with an anti-GFP ab. (B) Cultured hippocampal neurons (7 DIV) were transfected with myc-Kv2.1-Nav1.2 1080-1203. 24 h post-transfection, neurons were immunostained for myc, ankG and Nav1.2 (left panel), and for myc, ankG and CK2α (right panel). Scale bar = 20 µm. (C) The dominant negative effect of Kv2.1-Nav1.2 1080-1203 on ankG and Nav1.2 AIS localization was determined by quantifying the immunofluorescence intensity of ankG and Nav1.2 in transfected and non-transfected neurons (NT). Intensities were normalized to the control condition (n = 32 neurons from three independent experiments). Bars indicate mean ± SEM. One-way ANOVA post-test comparison, $^{***}P < 0.001$. (D) The dominant negative effect on Kv2.1-Nav1.2 1080-1203 or Kv2.1-Nav1.2 4SA on CK2 AIS localization was determined by quantifying the immunofluorescence intensity of CK2 in transfected and non-transfected neurons (NT). Intensities were normalized to the control condition (n = 39–86 neurons from three independent experiments). Bars indicate mean ± SEM. One-way ANOVA post-test comparison, $^{***}P < 0.001$.

mutated to alanines. This phospho-deficient chimera (Kv2.1-Nav1.2 4SA) did not displace endogenous Nav1 from the AIS, even though it is highly clustered at the AIS (data not shown and [7]), and had no effect on CK2 localization at the AIS (Fig. 3D). All these observations strongly suggest that CK2 forms a molecular complex with Nav1 at the AIS, allowing for its clustering.

4. Discussion

It has been well documented that the precise clustering of Nav1 at the AIS and nodes of Ranvier in myelinated axons is crucial for normal physiology of neurons. To date, we are starting to understand how Nav1 clustering is regulated in vitro and in vivo. Recent studies showed that an intact ABM is both necessary and sufficient for clustering of the full-length Nav1.6 to both the AIS and nodes of Ranvier [17], and that CK2-mediated phosphorylation of ABM facilitates ankG-Nav1 interaction [7]. In the present study, we evaluated the in vivo existence of CK2-mediated phosphorylation of ABM and the mechanism responsible for CK2 accumulation at the AIS. We showed, for the first time, using phosphospecific antibodies, that the CK2-dependent serine of ABM is phosphorylated in vivo during neuronal development as well as in adult rat brain. We demonstrated that the silencing of either Nav1 or CK2 in immature neurons disturbs both accumulations at the AIS. Finally, GST pull-down assays combined with dominant-negative experiments show that CK2α interaction with Nav1 intracellular loop II-III, is necessary for its AIS localization. Thus, our study reveals that CK2-mediated phosphorylation of Nav1 ABM occurs in vivo and that CK2 efficient accumulation at the AIS is regulated *via* its interaction with Nav1.

It has been reported that sub-populations of CK2 may be regulated through particular protein–protein interactions that may play a role in recruiting CK2 into the vicinity of its substrates and/or modulating its ability to be targeted to specific cellular domains [8]. Here, we showed that CK2 interacts with the intracellular loop II-III of Nav1.2, and that any displacement or early

inhibition of Nav1 expression impairs CK2 accumulation at the AIS. This suggests that CK2 binds directly or *via* a yet unidentified scaffold protein to Nav1, to regulate its ability to be specifically targeted to the AIS. Also, this interaction requires the presence of the entire loop II–III. However, we cannot conclude where this interaction occurs and if this interaction is constitutive or not. A recent study showed that the transport of Nav1 channels into axons is dependent on the complex KIF5-ankG, where ankG functions as an adaptor to link Nav1 channels to KIF5 [18]. All together, these observations suggest that CK2 could bind to Nav1 molecular complex early in the lifetime of Nav1 channels. Thus, it is likely that early Nav1-anchored CK2 regulates the probability of interaction with ankG *via* the phosphorylation of ABM to enhance Nav1 axonal transport and/or controls its specific transport to the AIS to promote its activity in this subcellular compartment. Similarly, KCNQ2 (Kv7.2) channels, which are clustered at the AIS, anchor CK2 and phosphatase PP1 [19]. This molecular complex provides a tonic regulation of the phosphorylation of calmoduline (CaM) in the vicinity of KCNQ2 subunits. CK2-mediated phosphorylation of CaM regulates the M-current by facilitating CaM binding to KCNQ2 [19]. Thus, it appears that the existence of CK2 in target protein complexes, like ion channels, could be a fundamental mechanism to regulate CK2 specific activity at the AIS.

In conclusion, we demonstrated that phosphorylation of ABM occurs in vivo and that CK2 accumulation at the AIS is regulated via its interaction with Nav1 complexes. This pathway may present an important regulatory mechanism to control neuronal excitability.

Author contributions

Y.E.H. and A.M. contributed equally to this work as well as H.V. and B.D; H.V. and B.D. designed experiments; F.C., O.F. and C.L. generated reagents and analytic tools; Y.E.H. and A.M. performed experiments; H.V. and B.D. wrote and edited the manuscript.

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