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IκBα is not required for axon initial segment assembly

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Abstract

The inhibitor of NF-κB alpha (IκBα) protein is an important regulator of the transcription factor NF-κB. In neurons, IκBα has been shown to play a role in neurite outgrowth and cell survival. Recently, a phosphorylated form of IκBα (pIκBα Ser32/36) was reported to be highly enriched at the axon initial segment (AIS) and was proposed to function upstream of ankyrinG in AIS assembly, including ion channel recruitment. However, we report here that the AIS clustering of ankyrinG and Na^+ channels in the brains of IκBα knockout (*Nfkbia*$^{-/-}$) mice is comparable to that in wild-type littermates. Furthermore, we found that multiple phospho-specific antibodies against pIκBα Ser32/36 non-specifically label AIS in *Nfkbia*$^{-/-}$ cortex and AIS in dissociated *Nfkbia*$^{-/-}$ hippocampal neurons. With the exception of ankyrinG, shRNA-mediated knockdown of known AIS proteins in cultured hippocampal neurons did not eliminate the AIS labeling with pIκBα antibodies. Instead, the pIκBα antibodies cross-react with a phosphorylated epitope of a protein associated with the microtubule-based AIS cytoskeleton that is not integrated into the AIS membrane complex organized by ankyrinG. Our results indicate that pIκBα is neither enriched at the AIS nor required for AIS assembly.

Keywords

Axon initial segment; ankyrinG; NF-κB signaling pathway; neuronal polarity

Introduction

The axon initial segment (AIS) is the site of action potential (AP) initiation in neurons (Khaliq & Raman, 2006; Kole *et al.*, 2008; Hu *et al.*, 2009; Foust *et al.*, 2010; Palmer *et al.*, 2010; Popovic *et al.*, 2011). High-density clusters of voltage gated Na^+ channels facilitate action potential (AP) initiation at the distal AIS and are recruited by the cytoskeletal adaptor protein ankyrinG (ankG) (Catterall *et al.*, 1981; Zhou *et al.*, 1998; Jenkins & Bennett, 2001; Garrido *et al.*, 2003; Lemaillet *et al.*, 2003; Kole *et al.*, 2008; Lorincz & Nusser, 2010). Recent reports show that the AIS cytoskeleton plays an important role in AIS assembly and long-term maintenance (Song *et al.*, 2009; Tapia *et al.*, 2010; Leterrier *et al.* 2011; Maniar *et al.*, 2011; Sanchez-Ponce *et al.*, 2011). For example, the AIS cytoskeleton stabilizes AIS membrane proteins and acts as a molecular sieve to maintain axodendritic polarity

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(Winckler *et al.*, 1999; Song *et al.*, 2009). In particular, ankG is required for AIS formation and maintenance (Hedstrom *et al.*, 2008; Sobotzik *et al.*, 2009), yet little is known about the proteins that act upstream of ankG in AIS assembly (Rasband, 2010).

Among the proteins enriched at the AIS, pIκBα was the first to be proposed to act upstream of ankG in AIS formation (Sanchez-Ponce *et al.*, 2008). IκBα is an inhibitor of the transcription factor NF-κB. Phosphorylation of IκBα at serines 32 and 36 frees NF-κB to traffic to the nucleus and initiate transcription (Viatour *et al.*, 2005). NF-κB signaling is critically involved in cell growth, and survival and IκBα has been shown to specifically contribute to neurite outgrowth and neuronal plasticity (Chen *et al.*, 2001; Gutierrez *et al.*, 2005). Schultz *et al.* (2006) reported the enrichment of multiple NF-κB signaling pathway proteins at the AIS. Interestingly, only phosphorylated forms of these proteins (phospho-IκBα (Ser32/36), phospho-IKKα/β (Ser180/Ser181), phospho-NF-κB p65 (Ser536)) were shown to be enriched at the AIS by immunfluorescence. However, these findings were somewhat surprising since IκBα phosphorylated at serines 32 and 36 is reported to be immediately targeted for degradation by the ubiquitin proteasome system (Karin & Ben-Neriah, 2000). Nonetheless, subsequent pharmacological studies using IKKα/β inhibitors in hippocampal neuron cultures revealed a possible role for pIκBα in the recruitment of ankG and voltage-gated ion channels to the AIS (Sanchez-Ponce *et al.*, 2008; Sanchez-Ponce *et al.*, 2010).

In light of pIκBα's reported role in AIS formation (Sanchez-Ponce *et al.*, 2008), we examined AIS assembly and neuronal development in IκBα null (*Nfkbia*$^{-/-}$) mice (Beg *et al.*, 1995). Unexpectedly, *Nfkbia*$^{-/-}$ brains had normal AIS, with ankG and Na^+ channel enrichment at the proximal axon in early development. More surprisingly, immunostaining with three independent phospho-specific antibodies targeting pIκBα (Ser32/36) in *Nfkbia*$^{-/-}$ tissue and dissociated hippocampal cultures derived from IκBα knockout mice showed that the AIS signal for pIκBα is not specific. Instead, the antibodies detect a phosphorylated epitope of a protein associated with the microtubule-based cytoskeleton at the AIS. The off-target AIS protein does not colocalize with ankG at the membrane, yet it depends on ankG for its AIS accumulation. Our results demonstrate that pIκBα is not required for AIS assembly and the pIκBα antibodies that detect the AIS by immunostaining recognize an unidentified protein associated with the microtubule-based cytoskeleton at the AIS.

Results

AIS assembly is normal in IκBα knockout mice

Inhibiting the phosphorylation of IκBα was reported to disrupt AIS assembly and axon outgrowth in cultured hippocampal neurons (Sanchez-Ponce *et al.*, 2008). To study the role of pIκBα in AIS assembly *in vivo*, we examined the subcellular localization of ankG and voltage-gated Na^+ channels in IκBα knockout (*Nfkbia*$^{-/-}$) mouse brain (Beg *et al.*, 1995). *Nfkbia*$^{-/-}$ mice die by P9; therefore, we performed our studies on P6 mice. All mice used in experiments were genotyped (Fig. 1A), and immunoblot analysis of brain membrane homogenates from littermates of each *Nfkbia* genotype confirmed the absence of IκBα protein expression in the *Nfkbia*$^{-/-}$ animals (Fig. 1B). However, immunolabeling of P6 brain tissue revealed no difference in AIS formation between control and *Nfkbia*$^{-/-}$ mice. Specifically, we found no statistically significant difference in ankG and pan-Na^+ channel immunofluorescence intensity at P6 cortical neuron AIS between genotypes [AnkG FI (AU): *Nfkbia*$^{+/+}$ 92.84±10.3, *Nfkbia*$^{+/-}$ 87.2±5.1, *Nfkbia*$^{-/-}$ 89.0±6.7 (n=10), p=0.87; Na_v FI (AU): *Nfkbia*$^{+/+}$ 101.2±11.7, *Nfkbia*$^{+/-}$ 123.2±6.6, *Nfkbia*$^{-/-}$ 124.6±7.8 (n=10), p=0.14] (Fig. 1C). Similarly, AIS length in cortical layer II/III neurons did not differ significantly between genotypes [AIS length (μm): *Nfkbia*$^{+/+}$ 26.4±1.7, *Nfkbia*$^{+/-}$ 25.0±1.5, *Nfkbia*$^{-/-}$ 24.9±1.6 (n=10), p=0.75]. Since pIκBα immunosignal was also previously reported at a

subset of nodes of Ranvier (Politi *et al.*, 2008), we also examined node formation in *Nfkbia*$^{-/-}$ tissue. However, like the AIS, no differences in node formation or structure were observed among genotypes (Fig. 1D). Together, our data indicate that IκBα is not required for assembly of the AIS or nodes of Ranvier.

AIS localization of pIκBα depends on ankyrinG expression

Since ankG and Na^+ channels become enriched at the AIS in the absence of IκBα expression, we considered whether the AIS localization of pIκBα depends on ankG. In hippocampal neurons, pIκBα antibody immunoreactivity is highly enriched at the AIS (Schultz *et al.*, 2006; Sanchez-Ponce *et al.*, 2008; Sanchez-Ponce *et al.*, 2010) (Figs. 2A,B). Consistent with previous reports (Schultz *et al.*, 2006; Sanchez-Ponce *et al.*, 2008), we found that pIκBα antibodies strongly label ankG-positive AIS following detergent extraction of dissociated hippocampal cultures (Fig 2C). Since proteins that remain at the AIS following detergent extraction are thought to directly interact with the detergent-resistant AIS cytoskeleton organized by ankG, we decided to test whether pIκBα clustering at the AIS depends on ankG. To this end, we silenced ankG expression during early development by transfecting ankG-targeted shRNAs into cultured hippocampal neurons at the time of plating. We found that pIκBα immunoreactivity was not enriched at the AIS of neurons lacking ankG (Fig. 2D). Therefore, the AIS-localization of pIκBα requires ankG. Furthermore, we found that pIκBα also depends on ankG for its long-term maintenance at the proximal axon. Silencing ankG expression by shRNA-mediated knockdown in mature neurons resulted in the subsequent loss of AIS pIκBα immunosignal (Fig. 2E).

Though multiple pIκBα antibodies clearly label the AIS, phosphorylation-independent IκBα antibodies do not show IκBα enrichment at the AIS; rather, these antibodies provide a faint and diffuse labeling of both the somatodendritic and the entire axonal compartment (including the AIS) (Fig. 2F). To confirm that IκBα, regardless of its phosphorylation state, is recruited to and enriched at the AIS, we took advantage of the characteristic detergent-insolubility of the AIS protein complex (Winckler *et al.*, 1999). Proteins associated with the ankG-organized membrane complex of the AIS and the local cytoskeleton are resistant to extraction by 1% TX-100 (Winckler *et al.*, 1999; Boiko *et al.*, 2007; Bréchet *et al.*, 2008; Sanchez-Ponce *et al.*, 2008; Tapia *et al.*, 2010). By contrast, somatodendritic proteins are easily solubilized in 1% TX-100 extraction buffer. Exploiting this property of AIS associated proteins, we hypothesized that if IκBα is enriched at the AIS, then both pIκBα and phosphorylation-independent IκBα immunoreactivity should be detectable at the AIS following detergent extraction. In stark contrast to the clear co-localization of pIκBα immunosignal with ankG following detergent extraction (Fig. 2C), immunostaining with the phosphorylation-independent IκBα antibody was completely abolished by detergent extraction (Fig. 2G). This discrepancy suggested that the AIS antigen detected by antibodies targeting pIκBα (Ser32/36) may not be IκBα, but some other antigen.

pIκBα AIS immunoreactivity is non-specific

To directly test the specificity of the pIκBα antibodies, we co-immunostained *Nfkbia*$^{-/-}$ brain tissue with antibodies against ankG and one of three independent, commercially available pIκBα (Ser32/36) antibodies. Surprisingly, these ‘phospho-specific IκBα’ antibodies clearly labeled ankG-positive AIS in tissue lacking IκBα expression (Fig. 3A). When we performed a Western blot analysis of brain homogenates made from *Nfkbia*$^{-/-}$ brains, we saw a strong band at an IκBα-like molecular weight (~ 41kDa) further confirming the non-specificity of the phospho-IκBα antibodies (Fig. 3B). Finally, AIS immunoreactivity from the pIκBα antibodies was also detected in hippocampal neurons cultured from P0 *Nfkbia*$^{-/-}$ mice (Fig. 3C). Line scans show comparable pIκBα antibody AIS-immunoreactivity in *Nfkbia* wild-type, heterozygous, and knockout hippocampal

neurons (Fig. 3C). Taken together, our results strongly support the conclusion that pIκBα is not required for AIS assembly and that pIκBα is not enriched at the AIS.

AIS antigen is not directly associated with ankyrinG

It is possible that the pIκBα antibodies recognize a protein previously reported at the AIS. To test whether the non-specific pIκBα (Ser32/36) antibodies cross-react with known AIS proteins, we transfected cells with shRNA constructs to silence the expression of several AIS proteins. The efficacy of each shRNA construct was validated previously (Hedstrom *et al.*, 2007). We also confirmed protein knockdown by immunostaining for the target protein in parallel with pIκBα immunostaining. pIκBα antibody immunoreactivity was still detected at the AIS of hippocampal neurons lacking Na^+ channels, βIV spectrin, NrCAM, or neurofascin-186 (Nfasc186) (Fig. 4A). Thus, the pIκBα antibodies do not cross-react with any of these AIS proteins. Knockdown of ankG, however, eliminated pIκBα immunoreactivity within the proximal axon (Figs. 4A, 2F–G).

Schultz *et al.* (2006) demonstrated that pIκBα antibody immunosignal is centrally located within the AIS and is surrounded by AIS membrane protein staining, suggesting an association with the microtubule-based cytoskeleton. To confirm that the pIκBα antibodies do not cross-react with ankG, we overexpressed ankG in cultured hippocampal neurons. Interestingly, we found that the pIκBα antibody immunosignal remained restricted to the interior of the AIS and was not present in the small membrane protrusions that can be seen after ankG overexpression (Fig. 4B). In contrast, Nfasc186 (a direct binding partner of ankG) immunoreactivity co-localized with ankG in the AIS membrane protrusions (Fig. 4B). The absence of pIκBα antibody immunoreactivity in the ankG-positive AIS protrusions demonstrates that the antigen detected by pIκBα antibodies is not ankG. Further, these results suggest that it is not a direct binding partner of ankG but instead is associated with the AIS cytoskeleton.

Non-specific pIκBα antibodies recognize a phosphorylated, MT-associated AIS protein

To begin to identify the AIS protein recognized by the non-specific pIκBα (S32/36) antibodies, we first confirmed that the protein of interest is indeed phosphorylated. We found that the pIκBα AIS immunosignal is abolished by alkaline phosphatase treatment of neurons permeabilized prior to fixation (Fig. 5A). Next, we treated the cells with the microtubule depolymerizing agent Nocodazole for up to 24h prior to fixation. Within two hours of Nocodazole treatment, there was a detectable disruption of pIκBα antibody immunofluorescence signal at the AIS (Fig. 5B). At the 24h time-point, pIκBα immunoreactivity was completely absent from the ankG-positive AIS (Fig. 5B). This finding is consistent with previous reports (Schultz *et al.*, 2006). In contrast, actin depolymerization by cytochalasin-D or latrunculin-B treatment had no effect on pIκBα antibody AIS fluorescence intensity (Fig. 5C). Similar results were obtained with a second pIκBα (Ser32/36) antibody (Fig. S1). Therefore, the AIS protein detected by the pIκBα antibodies is phosphorylated and is associated with the microtubule-based cytoskeleton at the AIS.

Discussion

Neuronal activity is governed by the AIS. In turn, neuronal activity influences gene regulation. However, neither the molecular mechanisms governing AIS assembly nor the relationship between activity and transcription are well understood (Rasband 2010). Therefore, it was very exciting when components of the NF-κB signaling pathway were reported at the AIS, making it easy to postulate a link between AIS activity and gene regulation (Schultz *et al.*, 2006). In addition, pIκBα was reported to be the first signaling molecule to regulate ankG clustering (Sanchez-Ponce *et al.*, 2008). However, the data

presented here argue that pIκBα is dispensable for ankG clustering and AIS assembly. Instead, we demonstrate that the antigen detected by the phospho-specific IκBα antibodies at the AIS is not phosphorylated IκBα but instead is an as yet unidentified, but phosphorylated, AIS protein associated with the local microtubule-based cytoskeleton. Our attempts to isolate and identify the AIS protein detected by the pIκBα antibodies by immunoprecipitation and mass-spectrometry have thus far proven unsuccessful (data not shown). We speculate this is due to the strong detergent-resistant nature of the proteins at the AIS (Boiko *et al.*, 2003) (Figs. 2C, E).

Our results underscore the importance of stringent control experiments when using antibodies that are reportedly 'phospho-specific.' Control experiments must include the use of mice that lack the target antigen (Rhodes & Trimmer, 2006; Lorincz & Nusser, 2008). Although our results exclude pIκBα from the AIS, we cannot rule out other members of the NF-κB signaling pathway as components of the AIS. For example, antibodies targeting phosphorylated IKKβ show AIS immunoreactivity and application of the IKKβ inhibitor BMS 345541 disrupts AIS formation *in vitro* without interfering with axon growth (Schultz *et al.*, 2006; Sanchez-Ponce *et al.*, 2008). Since activated IKKβ is the kinase that phosphorylates IκBα, the latter finding is seemingly contradictory to our report here that pIκBα is dispensable for AIS formation both *in vivo* and *in vitro*; however, it is possible that the interpretation of the IKKβ inhibition study results may have been confounded by the off-target effects of BMS 345541 on additional kinases such as ERK8, PKD1, CK1, or CDK2 (Bain *et al.*, 2007). Future experiments must confirm the specificity of the phospho-specific antibodies designed against proteins in the NF-κB signaling pathway. The importance of such experimental rigor is further highlighted by the recent findings of Herkenham *et al.* (2011) who demonstrate the non-specificity of many commonly used commercial antibodies targeting the NF-κB subunit proteins p65 and p50.

Our findings are consistent with the emerging concept that phosphorylation of AIS ion channels, their auxiliary subunits, and local cytoskeletal proteins may regulate AIS assembly and function (Bréchet *et al.*, 2008; Sanchez-Ponce *et al.*, 2010 Leterrier *et al.*, 2011; Li *et al.*, 2011; Vacher *et al.*, 2011). Consistent with this idea, several kinases have now been reported at the AIS. For example, phosphorylation of Na^+ channel α subunits by CK2 at the AIS was reported to promote their interaction with ankG (Bréchet *et al.*, 2008). Cdk-mediated phosphorylation of Kvβ2 at the AIS controls the release of $K_v\beta2$-K_v1 K^+ channel complexes from the microtubule-associated end binding protein EB1 for insertion within the AIS membrane (Vacher *et al.*, 2011). Moreover, CamKII was shown to be enriched at Purkinje neuron AIS where it interacts with βIV spectrin (Hund *et al.*, 2010). Thus, the AIS may be a 'hotspot' for phosphorylation, which could explain why only phospho-antibodies against NF-κB signaling proteins labeled the AIS (Schultz *et al.*, 2006).

Several recent reports demonstrate a previously unrecognized plasticity in AIS structure that is correlated with changes in neuronal excitability (Grubb & Burrone, 2010; Kuba *et al.*, 2010; Kaphzan *et al.*, 2011). By analogy to the phosphorylation-dependent events that facilitate synaptic remodeling (Evers *et al.*, 2010; Lee *et al.*, 2011), plastic changes at the AIS may also depend on local protein phosphorylation. Future studies to identify the enzymes that regulate dynamic processes at the AIS, including ion channel stability and availability, cytoskeletal dynamics, and axonal trafficking, will improve our understanding of the multi-functional role of the AIS in neuronal development, excitability, and plasticity.

Experimental Methods

Animals

Nfkbia$^{+/-}$ mice were described previously (Beg *et al.*, 1995) and generously provided by Dr. Hui Zheng, Baylor College of Medicine. *Nfkbia*$^{+/-}$ mice were crossed to obtain *Nfkbia*$^{+/-}$, *Nfkbia*$^{-/-}$, and *Nfkbia*$^{+/+}$ mice. Wild-type C57BL/6 mice were purchased from Jackson Laboratories. Timed-pregnant Sprague Dawley rats were purchased from Harlan Sprague Dawley. Animals were housed and maintained in Baylor College of Medicine's Center for Comparative Medicine, compliant with the NIH Guide for Care and Use of Laboratory Animals.

Genotyping

Genotyping was performed by PCR of tail snip DNA from either P0 or P6 mice, per experimental requirements. A three-primer multiplex from *Nfkbia* was used, including a common forward primer (AGTGGCTCATCGCAGGGAGTTTCT), a reverse wild-type (CAGCTCCTTCACCATTTGCTCGTA), and a reverse knockout primer (CGGTATCGATACTGGCTGAA).

Antibodies

The following primary antibodies were used: rabbit polyclonal anti- IκBα (C21) (SC-371, Santa Cruz Biotechnology), mouse monoclonal anti-phospho-IκBα (Ser32/36) (5A5, Cell Signaling Technology), rabbit monoclonal anti-phospho-IκBα (Ser32/36) (14D4, Cell Signaling Technology), rabbit polyclonal anti-phospho-IκBα (Ser32/36) (SC-101713, Santa Cruz Biotechnology), mouse monoclonal anti-ankG (N106/36, UC Davis/NIH NeuroMab Facility), chicken and rabbit polyclonal anti-βIV spectrin (Yang *et al.*, 2004), mouse monoclonal anti-pan-Na^+ channel (K58/35, Sigma), rabbit polyclonal anti-Caspr (Schafer *et al.*, 2004), rabbit polyclonal anti-GFP (Invitrogen), chicken polyclonal anti-MAP2 (Encor Biotechnology Inc.), and mouse monoclonal anti-pan-neurofascin (L11A/41.6, Schafer *et al.*, 2004). Alexa-fluorophore-conjugated secondary antibodies were purchased from Invitrogen. AMCA-conjugated goat anti-chicken secondary antibody was purchased from Jackson Immuno Research.

Primary neuron culture

Dissociated hippocampal neurons were cultured essentially as in Kaech & Banker (2006), in the absence of the glial feeder layer. In brief, hippocampi were isolated from the brains of either E17 or P0 mouse embryos or E18 rat emrbyos, trypsin-digested (0.25% trypsin in HBSS), and dissociated by trituration prior to plating on poly-L-lysine and laminin-coated coverslips. Neuronal growth media (97% Neurobasal, 2% B-27 supplement, 1% Glutamax. Invitrogen) was supplemented, in part, with new media every four days.

Transfection of cultured neurons

Immediately following dissection and dissociation, neurons were nucleofected with shRNA or cDNA expression plasmids using the Neon system (Invitrogen). The neurons were suspended in 5ml HBSS and centrifuged 5 minutes at 1300rpm. The supernatant was removed and the cell pellet was subsequently resuspended in resuspenion buffer T at a density of 24,000 cells/μl. A single 1400mV pulse was delivered over 20ms to electroporate the cell membrane and introduce shRNA or cDNA plasmids. Ten microliters of suspended, electroporated cells were plated on poly-L-lysine and laminin-coated coverslips. Media was completely replaced at 4h following transfection. Hippocampal neurons were maintained from one to three weeks as indicated in primary neuron cultures methods section. The efficacy of the Na^+ channel, Nfasc186, NrCAM, and βIV spectrin shRNA expression

plasmids was reported in Hedstrom *et al.* (2007). The GFP-tagged ankG cDNA expression plasmids were a gift from Dr. Vann Bennett, Duke University.

Viral transduction of cultured neurons

Adenovirus-mediated RNA interference was used to silence ankG expression in either developing (0 DIV) or mature (11 DIV) cultured hippocampal neurons. At the indicated time points, cells were incubated 4h in media containing virus which was then exchanged completely for virus-free media. Adenoviruses containing ankG shRNA expression plasmids were generated by Hedstrom *et al.* (2008). Cells were maintained in culture either seven to ten days following infection prior to fixation.

Cytoskeletal depolymerizing drug treatments

Dissociated hippocampal neuron cultures were maintained three weeks at which point the media was supplemented with either 10μM Nocodazole, 20μM cytochalasin-D, 20μM latrunculin-B, or vehicle (DMSO alone). Cells were fixed with ice-cold 4%-PFA at various drug incubation time points including two, six, and 24h and immunostained.

Immunostaining

Mice were deeply anesthetized with isoflurane before transcardial perfusion with ice-cold 4% PFA in 0.1M Na^+-phosphate buffer (PB), pH7.2. Brains were post-fixed in 4% PFA 0.1M PB for 1 hour and equilibrated in 20% sucrose 0.1M PB over 48 hours. Afterward, 25 μm coronal slices containing the hippocampal formation were cut on a microtome and washed in 0.1M PB. Slices were blocked in 10% normal goat serum 0.1M PB containing 0.3% TX-100 (PBTgs). Tissue was incubated overnight at 4°C in primary antibodies diluted in PBTgs. Primary antibodies were removed by washing the tissue 3 times for 5 minutes with PBTgs. Secondary antibodies diluted in PBTgs and applied for 1 hour at RT to visualize primary antibodies. Excess secondary antibodies were removed by consecutive 5-minute washes with PBTgs, 0.1M PB, and 0.05M PB. Slices were mounted on gelatin-coated coverslips.

Detergent extraction

Culture media was replaced with an equivalent volume of pre-warmed (37°C) detergent extraction (DE) buffer (2mM $MgCl_2$, 10mM EGTA, 60mM PIPES, 1% TX-100) and returned to the 37°C incubator for 8 minutes. The original DE buffer was aspirated and fresh, pre-warmed DE buffer was added for a second and third extraction. After the third incubation, DE buffer was removed and the cells were immediately fixed with ice-cold PFA.

Alkaline phosphatase treatment of cultured neurons

Culture media was replaced with an equivalent volume of pre-warmed (37°C) DE buffer and the cells were returned to the incubator for 8 minutes. Following permeabilization, the DE buffer was removed and immediately replaced with pre-warmed alkaline phosphatase (AP) diluted to 100U/ml in AP buffer (5mM $MgCl_2$, 100mM NaCl, 100mM Tris-HCl, pH9.5). Cells were returned to the incubator and the dephosphorylation reaction proceeded 20 minutes. The AP solution was aspirated and the cells were immediately fixed with ice-cold PFA.

Imaging

Fluorescence imaging was performed on an AxioImager Z1 microscope (Carl Zeiss MicroImaging) fitted with an AxioCam digital camera (Carl Zeiss MicroImaging). AxioVision acquisition software (Carl Zeiss MicroImaging) was used for collection of images. Comparison of WT, heterozygous *Nfkbia*$^{+/-}$, and knockout *Nfkbia*$^{-/-}$ tissue was

performed on slices prepared in parallel and images were acquired at identical exposure times. Experiments were performed at least in triplicate. Fluorescence intensity was measured using ImageJ (NIH). In some images, contrast and brightness were subsequently adjusted in a linear fashion using Photoshop (Adobe). Any adjustments made to images from one genotype were made in all genotypes.

Western blotting analysis

Mouse brains were rapidly dissected and immediately homogenized in ice-cold buffer containing (in mM): 50 Tris-HCl, 64.1 $MgCl_2$, and 320 sucrose, supplemented with protease inhibitor cocktail (Sigma) and the phosphatase inhibitors Na-fluoride (10μM) and Na-orthovandate (10μM). Membrane proteins were isolated by centrifugation. After protein quantification by BCA assay (Pierce), protein samples were denatured in SDS sample buffer containing β-mercaptoethanol and heated to 95°C for 3 minutes. After heating the samples, 10mg of brain membrane protein was loaded onto 7.5% SDS-PAGE gels, resolved, transferred onto nitrocellulose membranes, and probed with the indicated primary antibodies in 5%-milk TBS using standard techniques. HRP-conjugated secondary antibodies were used to visualize primary antibodies and purchased from Invitrogen.

Measurement of AIS parameters

AIS length and protein fluorescence intensity values were determined by line-scan length and gray-value quantification in Image J (NIH). Images were collected at equivalent exposures and were not adjusted subsequent to acquisition.

Statistical analysis

Two-way ANOVA tests with Bonferroni correction were performed on the AIS length and ankG and Na^+ channel fluorescence intensity (FI) data sets comparing the three genotypes. Results are reported as mean±s.e.m.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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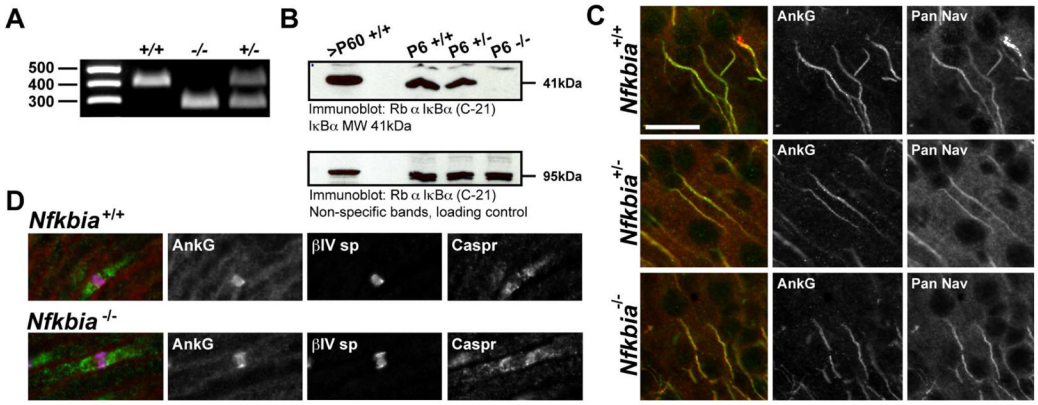

Figure 1.

Na^+ channel recruitment to the AIS and nodes of Ranvier is normal in *Nfkbia*$^{-/-}$ cortex, sciatic nerve. (A) Representative genotyping of littermates from *Nfkbia*$^{+/-}$ crosses used for immunofluorescence studies. (B) Western blot probed with phosphorylation-independent IκBα antibody showing lack of IκBα expression in the *Nfkbia*$^{-/-}$ knockout brain. Non-specific band from the same blot at 95 kDa is included as a loading control. (C) AnkG and pan-Na^+ channel AIS immunofluorescence intensity is similar between WT, *Nfkbia*$^{+/-}$ heterozygotes and *Nfkbia*$^{-/-}$ homozygous null cortex. Scale bar represents 20 μm. (D) Assembly of nodes of Ranvier is comparable between WT IκBα mice and IκBα knockouts.

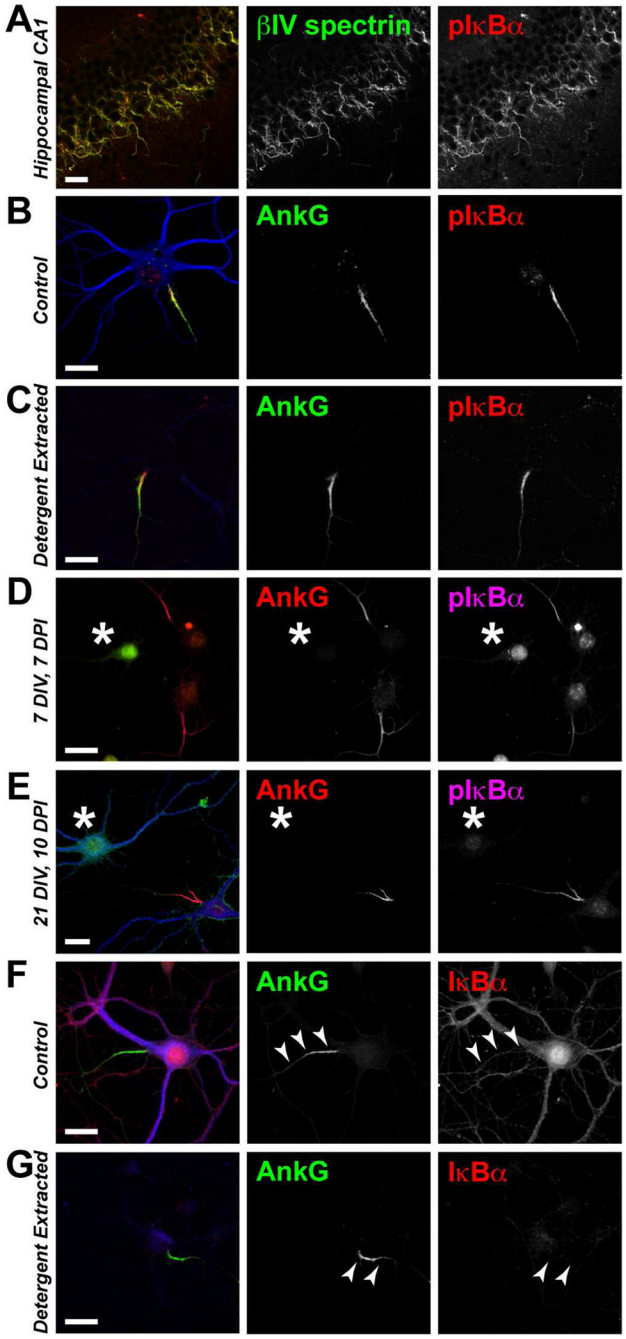

Figure 2.

Phospho(S32/36)-IκBα immunoreactivity is enriched at the AIS and depends on ankG expression. **A–C** (A) P6 wild-type mouse hippocampal CA1 stained for pIκBα and βIV spectrin. (B,C) Immunostaining of (B) control and (C) detergent-extracted cultured wild-type hippocampal neurons grown 21 DIV. **D, E** (D) Immunostaining of cultured hippocampal neurons transduced with ankG shRNA-containing adenovirus at the time of plating. (E) Immunostaining of cultured hippocampal neurons in which ankG expression was silenced by adenoviral ankG shRNA delivery at 11 DIV; cells were fixed at 21 DIV, 10 days post-infection (DPI). The transduced neurons are GFP-positive and indicated by an

asterisk. **F, G** Both (F) control and (G) detergent-extracted neurons show a lack of phosphorylation-independent IκBα clustering at the AIS (arrowheads). Scale bars represent 20μm.

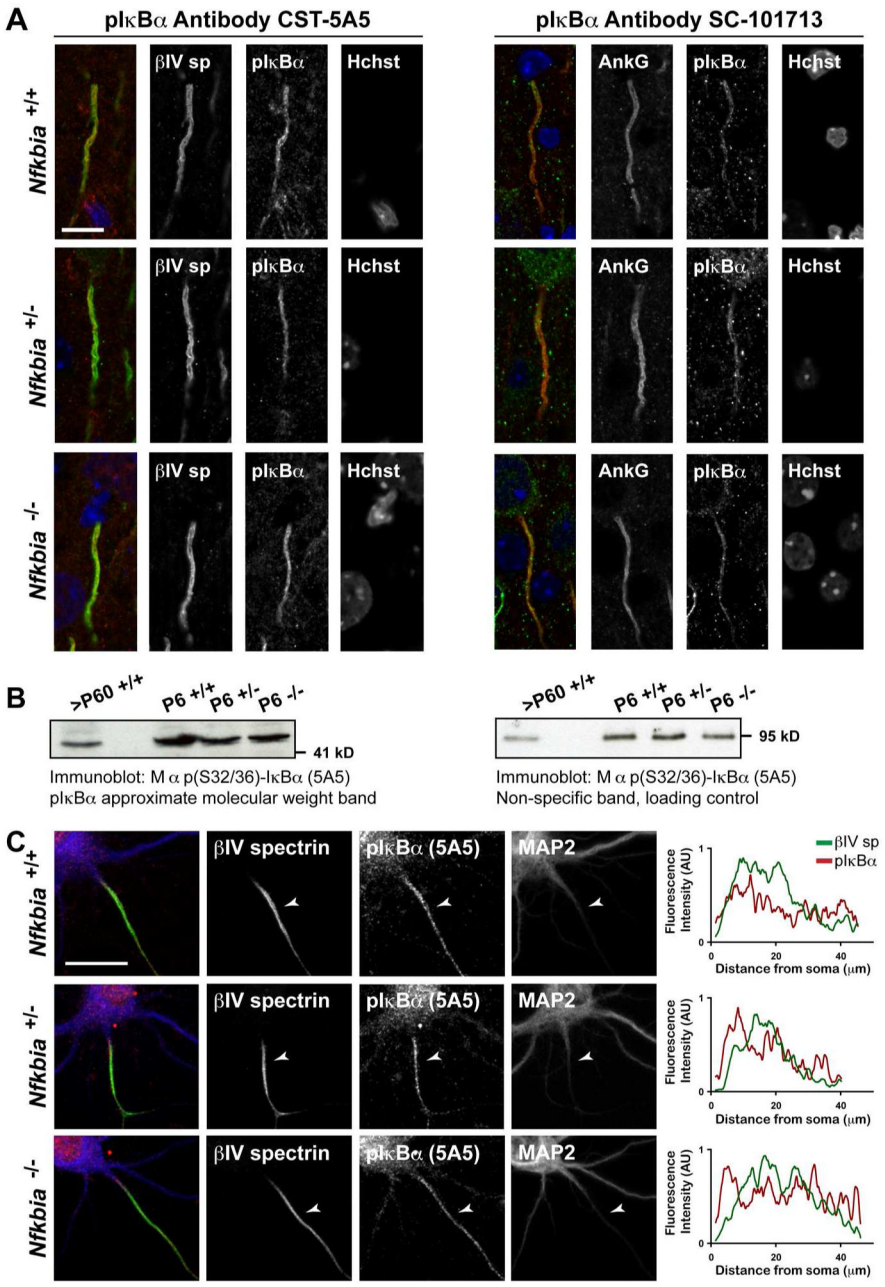

Figure 3.

Non-specific pIκBα immunoreactivity is evident at the AIS of *Nfkbia*$^{-/-}$ P6 brain tissue and cultured *Nfkbia*$^{-/-}$ hippocampal neurons. (A) P6 cortex stained for pIκBα and either βIV spectrin or ankG. Two independent antibodies designed to exclusively detect pIκBα (Ser32/36) demonstrate strong AIS immunoreactivity in IκBα KO neurons. Scale bar represents 10μm. (B) Western blot of homogenized brain membrane proteins from adult (>P60) and P6 *Nfkbia*$^{+/+, +/-,}$ and $^{-/-}$ mice probed with the monoclonal 5A5 pIκBα antibody. A non-specific band of 95 kDa is included as a loading control. (C) Hippocampal neurons cultured at P0 from *Nfkbia*$^{+/+, +/-,}$ or $^{-/-}$ littermates fixed and immunostained at 10 *DIV*. pIκBα immunoreactivity co-localizes with βIV spectrin at the AIS in neurons of all

genotypes. Fluorescence intensity plots show AIS line-scan fluorescence intensity data for each channel. Scale bar represents 20μm.

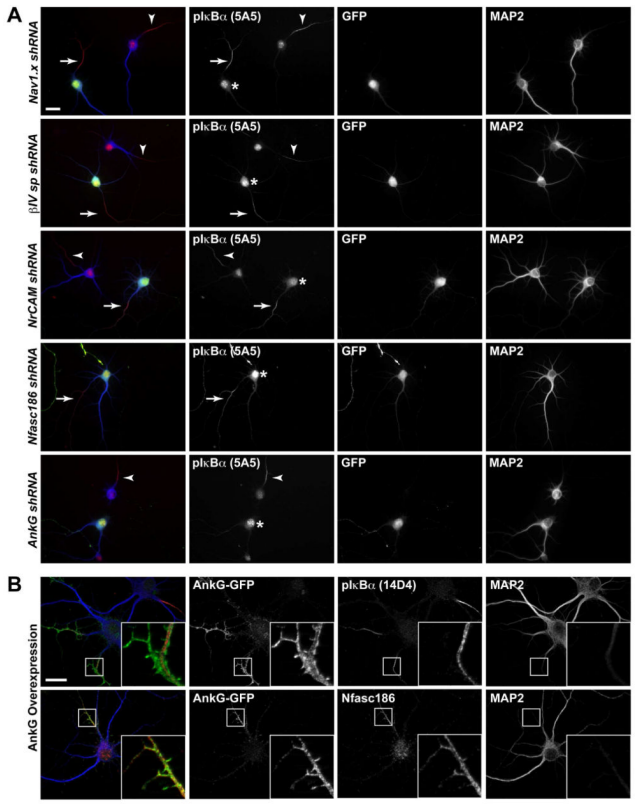

Figure 4.

pIκBα antibodies do not cross-react with known AIS proteins. (A) Immunostaining of 8 DIV cultured hippocampal neurons nucleofected with shRNA targeting either Na^+ channels, NrCAM, Nfasc186, βIV spectrin, or ankG, as indicated, at the time of plating. Transfected neurons express GFP. (B) Cultured hippocampal neurons transduced with ankG-GFP cDNA at the time of plating and immunostained at 15 DIV for ankG and either pIκBα (14D4) or Nfasc186. Scale bars represent 20μm.

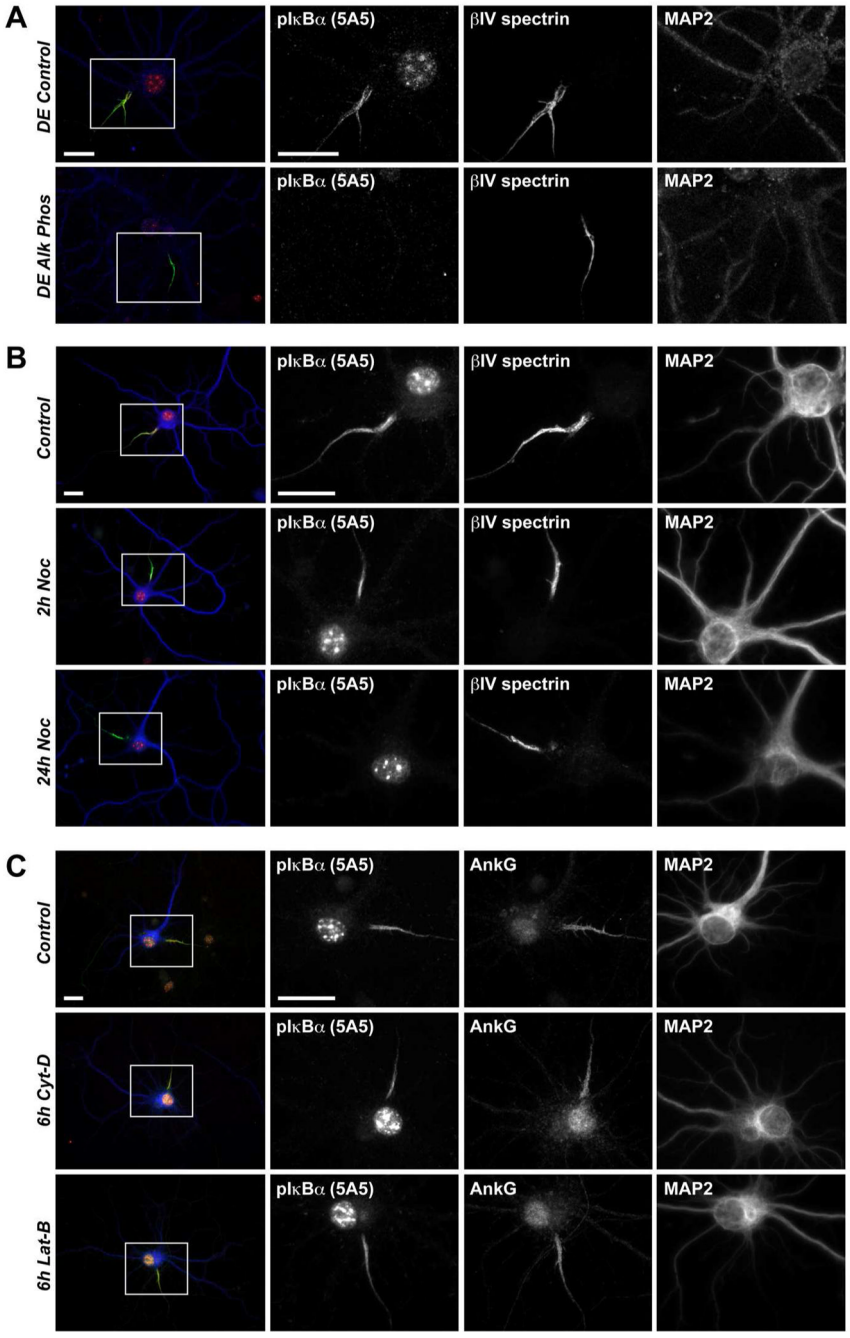

Figure 5.

pIκBα antibody 5A5 recognizes a phosphorylated protein associated with the microtubule-based AIS cytoskeleton. (A) Immunostaining of 14 DIV detergent-extracted hippocampal neurons treated with either control solution or alkaline-phosphatase for 20 minutes prior to fixation. (B) Immunostaining of 14 DIV cells treated with either DMSO alone for 24h or 25 μM Nocodazole, a microtubule depolymerizing drug, in DMSO over 2 or 24h. (C) Immunostaining of 14 DIV cells treated 6h with either control DMSO solution or 20 μM actin-depolymerizing agents Cytochalasin-D or Latrunculin-B. Scale bars represent 20 μm.