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PSD-93 CLUSTERS KV1 CHANNELS AT AXON INITIAL SEGMENTS INDEPENDENT OF CASPR2

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Abstract

PSD-93/Chapsyn-110 is a PDZ domain-containing membrane-associated guanylate kinase (MAGUK) that functions as a scaffold to assemble channels, receptors, and other signaling proteins at cell membranes. PSD-93 is highly enriched at synapses, but mice lacking this protein have no synaptic structural abnormalities, probably due to overlapping expression and redundancy with other MAGUKs. Consequently, the function of PSD-93 is not well understood. Here we show that PSD-93, but not other MAGUKs, is enriched at the axon initial segment (AIS) where it colocalizes with Kv1.1, Kv1.2, Kv1.4, and Kvβ2 subunit-containing K^+ channels, Caspr2, and TAG-1. When co-expressed with Kv1 channels in heterologous cells, PSD-93 induces formation of large cell-surface clusters. Knockdown of PSD-93 in cultured hippocampal neurons by RNA interference disrupted Kv1 channel localization at the AIS. Similarly, PSD-93 −/− mice failed to cluster Kv1 channels at the AIS of cortical and hippocampal neurons. In contrast, Caspr2, which mediates Kv1 channel clustering at the juxtaparanode is not required for localization of Kv1 channels at the AIS. These results show PSD-93 mediates AIS accumulation of Kv1 channels independently of Caspr2.

Keywords

action potential; juxtaparanode; scaffold; ankyrin; cell adhesion molecule; MAGUK

INTRODUCTION

Ion channels are enriched in neurons at the axon initial segment (AIS) and node of Ranvier where they generate, modulate, and propagate action potentials (Lai and Jan, 2006). Proper neuronal function requires that specific types of ion channels are restricted to discrete membrane domains. Voltage-gated sodium channels (Nav), KCNQ2/3 K^+ channels, neurofascin-186 (NF-186), ankyrinG (ankG), and βIV spectrin are highly enriched at the AIS and nodes (Hedstrom and Rasband, 2006). In myelinated axons, cell adhesion molecules (CAMs) cluster scaffolds and ion channels (Poliak and Peles, 2003; Eshed et al., 2005; Sherman et al., 2005). AIS clustering of Nav and KCNQ2/3 channels depends on binding to the scaffolding protein ankG, as these channels are depleted from the AIS of neurons lacking ankG

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(Zhou et al., 1998; Garrido et al., 2003; Lemaillet et al., 2003; Pan et al., 2006). Whereas the CAMs NF-186 and NrCAM cluster ion channels at nodes of Ranvier, they are dispensible for ion channel clustering at the AIS (Dzhashiashvili et al., 2007; Hedstrom et al., 2007).

Recently, Kv1.2 channels were reported at the AIS of human cortical pyramidal neurons (Inda et al., 2006), rat retinal ganglion cells (Van Wart et al., 2007), and avian nucleus laminaris neurons (Kuba et al., 2006). AIS Kv1.2 is more spatially restricted than ankG and is only in the distal segment of the AIS, suggesting ankG is not the sole determinant of Kv1 channel clustering (Van Wart et al., 2007). In myelinated axons, Kv1 channels flank nodes and are sequestered beneath the myelin sheath in juxtaparanodal membrane domains (Rasband, 2004). There, an axonal heterodimer consisting of the CAMs Caspr2 and TAG-1 participate in *trans*-interactions with glial TAG-1. These proteins are required for Kv1 channel localization (Poliak et al., 2003; Traka et al., 2003). Since Caspr2 and Kv1 channels both have PDZ binding motifs, they may be linked by a PDZ-domain scaffolding protein (Poliak et al., 1999). Indeed, PSD-95 localizes to juxtaparanodes and co-immunoprecipitates with Kv1 channels. However, mice lacking PSD-95 have normal juxtaparanodal Caspr2 and Kv1 channels (Rasband et al., 2002). Intriguingly, Caspr2 occurs at the AIS of human pyramidal neurons (Inda et al., 2006).

Here we report a macromolecular protein complex at the AIS composed of Kv1 channels, Caspr2, TAG-1, and the PDZ-domain containing scaffolding protein PSD-93/Chapsyn-110. We show that PSD-93 is necessary for Kv1 channel clustering at the AIS. However, in contrast to the juxtaparanode, Caspr2 is not required for localization of Kv1 channels to the AIS. Our results demonstrate that AIS and juxtaparanodal membrane domains share many common components, yet have distinct mechanisms that underlie their formation and maintenance.

MATERIALS AND METHODS

Animals

Sprague–Dawley rats were purchased from Charles River. Caspr2 −/− and PSD-93 −/− mice have been described previously (McGee et al., 2001; Poliak et al., 2003). Animals were housed at the Center for Laboratory Animal Care at the University of Connecticut Health Center and at the Weizmann Institute of Science. All experiments were performed in accordance with the National Institutes of Health guidelines for the humane treatment of animals.

Antibodies

Antibodies against Kv channel subunits and the Pan-Nav channel antibody have been described (Rhodes et al., 1995; Bekele-Arcuri et al., 1996; Rasband et al., 1999; Tiffany et al., 2000). Mouse monoclonal anti-PSD-93 (N18/30) and polyclonal anti-PSD-93 antibodies were obtained from NeuroMab (Davis, CA) and from Alamone labs (Israel), respectively. Additional rabbit polyclonal anti-PSD-95 and rabbit polyclonal anti-PSD-93 antibodies were kindly provided by Dr. Masahiko Watanabe (Fukaya and Watanabe, 2000). The mouse monoclonal anti-PSD-95 (K28/43.2) has been described (Rasband et al., 2002). Rabbit anti-βIV spectrin "specific" domain (SD) antibodies have been described (Berghs et al., 2000). Mouse monoclonal ankG antibodies were purchased from Invitrogen (San Francisco, CA). Rabbit polyclonal anti-Caspr2 antibody was purchased from United States Biological (MA). Chicken polyclonal anti-MAP2 antibody was purchased from (EnCor Biotechnology Inc, FL). Rabbit polyclonal antibodies against proteins 4.1G and 4.1N were kindly provided by Dr. Ilya Bezprozvanny (University of Texas Southwester, Dallas, TX). The rabbit polyclonal antibodies against protein 4.1B have been described (Gollan et al., 2002). Secondary antibodies used were Alexa-594 conjugated goat anti-mouse, Alexa-488 conjugated goat anti-rabbit (Invitrogen-

Molecular Probes, OR), and AMCA-conjugated goat anti-chicken (Jackson ImmunoReseach, PA).

Hippocampal neuron culture

Pregnant rats (E16–18) were deeply anesthetized with Halothane. Embryos were removed under sterile conditions, and brains were collected into ice-cold Hanks Buffered Salt Solution (HBSS; Invitrogen). Embryonic hippocampi were then dissected and collected in HBSS. These hippocampi were incubated with 0.25 % trypsin in HBSS at 37 °C for 15 minutes, and washed with HBSS. After adding plating media (Neurobasal medium (Invitrogen) supplemented with 10% FCS), these hippocampi were triturated gently using a fire-polished Pasteur pipette. The cell suspension was left for 5 minutes, and the supernatant was collected into a new tube to remove debris. Cells were then plated on cover glass coated with 1 mg/mL poly-D lysine and 20 μg/mL laminin at 2.5-3×10^5 cells/35 mm dish. Neurons were incubated in a humidified 5 % CO_2 incubator at 37 °C. The media was exchanged to maintenance media (Neurobasal medium with 2% B27 (Invitrogen), 0.5 mM L-glutamine and 25 μM L-glutamate) after 2–4 hours. On day 3, 1 μM cytosine arabinoside (Sigma) was added into culture media to inhibit proliferating cell growth. The cultures were maintained by exchanging half of the volume of media twice a week with new maintenance media.

shRNA and Transfection

For expression of shRNA, we subcloned into pBlueScriptII KS- (Stratagene, CA) a human H1 promoter derived from pSuper and 64-Mer oligonucleotides containing the sense and antisense of the target sequence linked by a hairpin loop of nine bases (TTCAAGAGA) and an added termination signal (TTTTT). The sequence of the sense strand of 19 nucleotides was chosen to target rat PSD-93 (RNU49049). Control shRNA: 5'-GCAUGUCACGAUGUUACAA-3'; r1 shRNA (bases 1159–1177): 5'-CACUCUUAUUCUCCACCAA-3'; and r3 shRNA (bases 2771–2789): 5'-GCAAGCUUGUUAUUGAAGA-3'. Hippocampal neurons were transiently co-transfected at 10–11 days after plating using Lipofectamine 2000 (Invitrogen, CA) to deliver an EF1α promoter driven GFP expression vector for identification of transfected neurons and the human H1 promoter driven shRNA expression vectors. Briefly, culture medium was removed until 1mL remained in the 35 mm dish. 0.5 μg of GFP plasmid and 1μg of pSUPER were mixed in 200 μL Neurobasal medium with 5 μL Lipofectamine 2000. This cocktail was added to each 35mm dish, and incubated at 37 °C for 4 hr before exchanging culture medium. Transfected neurons were analyzed 10 days later. For the clustering assay, recombinant plasmids were co-transfected into COS7 cells using lipofectamine 2000 according to the manufacturers instructions. The following plasmids were used: pGW-PSD-93EGFP (a gift from Dr. Bonnie Firestein, Rutgers, NJ), pGW-PSD95 (a gift from Dr. Morgan Sheng), RBG4-Kv1.1, RBG4-Kv1.2, RBG4-Kv1.4 (Nakahira et al., 1996), and p3XFLAG-Caspr2 (amino acids 1078–1332). Transfected cells were analyzed 18–24 hr after transfection by immunocytochemistry.

Immunostaining

Neurons were fixed with 1% paraformaldehyde (PFA) in PBS for 15 minutes at 4 °C. Brains were fixed by immersion with 4% PFA for 30 minutes at 4 °C, cryoprotected with 20 % sucrose, embedded in Tissue-Tek OCT mounting medium, and frozen on dry ice powder. Blocks were cut using a cryostat (Leica) to obtain 20 μm-thick sections, and the sections were placed on precoated slides (Fisher scientific). Cultured neurons or brain sections were blocked with 4 % non-fat dry milk powder in TBS (10 mM Tris-HCl; pH 7.5, 0.15 M NaCl) with 0.3% Triton X-100 for 30 minutes at room temperature. Primary antibodies diluted in the blocking buffer were added at appropriate concentrations, incubated at room temperature overnight, and washed with PBS. Secondary antibodies were incubated at room temperature for 1 hour and

washed with PBS. Fluorescence images were acquired using a Zeiss (Thornwood, NY) Axioskop 2 fluorescence microscope fitted with a Hamamatsu (Bridgewater, NJ) ORCA-ER camera.

Statistical analysis

The Mann-whitney *U* test was used to determine relationships between control shRNA and PSD-93 targeted shRNA transfected neurons, which were calculated by using StatView for Mac. All percentages given are median values for each measurement.

RESULTS

Multiple Kv1 channel subunits are located at the axon initial segment

The generation, modulation, and duration of axonal action potentials depend on both the high densities and the specific types of ion channels found at the AIS (Hedstrom and Rasband, 2006; Khaliq and Raman, 2006; Shu et al., 2006). Kv1 channels are prominent in axons and nerve terminals (Gu et al., 2003; Trimmer and Rhodes, 2003) and modulate action potentials (Kole et al., 2007). We used a panel of mouse monoclonal and rabbit polyclonal Kv1 channel subunit specific antibodies to determine the molecular composition of the AIS Kv1 channels in cultured rat hippocampal neurons. We used antibodies against βIV spectrin, Nav channels, and ankG to identify the AIS. Somatodendritic domains were defined by immunostaining with antibodies against MAP2. We found Kv1.1, Kv1.2, and Kv1.4 enriched at the AIS (Fig. 1 A–C) of neurons maintained for 11 days in vitro (DIV). We obtained identical results using Kv1 channel α-subunit specific antibodies from both rabbit and mouse (data not shown). Although Kv1.6 has been reported in mouse hippocampal cultures (Grosse et al., 2000), we were unable to detect any specific immunoreactivity (data not shown). We also found Kvβ subunits enriched at the AIS of many cultured hippocampal neurons (Fig. 1D and S1A). These same Kv1 channel α-subunits were also detected at the AIS of postnatal day 10 (P10) cortical neurons *in vivo* (Fig. 2). Thus, Kv1.1, Kv1.2, and Kv1.4 α-subunit-containing Kv channels are enriched at the AIS *in vitro* and *in vivo* where they likely exist as heteromultimeric complexes together with Kvβ subunits.

PSD-93 colocalizes with Kv1 channels at the AIS in vivo and in vitro

Localization and retention of Nav and KCNQ2/3 Kv channels at the AIS depends on a common intracellular domain that mediates channel interaction with the AIS scaffolding protein ankG (Garrido et al., 2003; Pan et al., 2006). How are Kv1 channels clustered at the AIS? Does their localization also depend on ankG or on some other scaffolding protein? Kv1.1, Kv1.2, and Kv1.4 each contain a consensus class I C-terminal PDZ (PSD-95/DLG/ZO-1) binding motif (xS/TxV), but not the ankG binding motif present in Nav and KCNQ channel subunits (Garrido et al., 2003; Lemaillet et al., 2003; Pan et al., 2006). PDZ binding motifs permit the interaction of Kv1 channels with PSD-95, a prominent scaffolding protein found at synapses (Kim et al., 1995). PSD-95 is the prototypical member of a family of membrane associated guanylate kinases (MAGUKs) or synaptic-associated proteins (SAPs) that include SAP97, PSD-95/SAP90, SAP102, and PSD-93/Chapsyn-110. These proteins are structurally similar with three PDZ domains, a src homology (SH3) domain, and a guanylate kinase (GK) domain (Sheng and Sala, 2001). Furthermore, MAGUKs colocalize and interact with Kv1 channels at other CNS locations including cerebellar pinceau (Kim et al., 1995; Laube et al., 1996) and juxtaparanodes of myelinated axons (Rasband et al., 2002). Therefore, we asked whether these proteins might regulate the localization of Kv1 channels at the AIS.

Immunostaining of 11 DIV cultured hippocampal neurons revealed PSD-93 signals at synapses (Fig. 3A). In addition, PSD-93 immunoreactivity was detected at the AIS where it colocalized with Nav channels (Fig. 3A, inset). In contrast, PSD-95 was detected only at synaptic sites and

not at the AIS (Fig. 3B). Double-immunostaining with antibodies against PSD-93 and PSD-95 revealed only PSD-93 signals at the AIS (Fig. 3C, inset), and double-immunostaining with antibodies against Kv1.2 and PSD-93 showed that these two proteins colocalized at the AIS (Fig. 3D). These observations *in vitro* agree with a previous report of PSD-93 at the AIS (Rao et al., 1998). Despite a recent report that SAP102 interacts with NrCAM, a CAM that is highly enriched at the AIS (Davey et al., 2005), we detected neither SAP102 nor SAP97 at the AIS (Figs. 3E and 3F, respectively). We also confirmed that PSD-93 is located at the AIS of P10 rat cortical neurons *in vivo* and that it colocalizes with ankG (Fig. 4A, arrows) and Kv1.2 (Fig. 4B, arrows).

PSD-93 and Kv1 channels make surface clusters in vitro

To demonstrate that Kv1 channels and PSD-93 interact, we performed a surface clustering assay by co-expressing Kv1 channels and PSD-95 or PSD-93 in COS7 cells (Kim et al., 1995, 1996a; Tiffany et al., 2000). We used an anti-Kv1.1 antibody directed against an extracellular domain (Kv1.1 ext) to detect surface Kv1.1 before permeabilizing the cells (Tiffany et al., 2000). Since the α-subunit composition of Kv1 channels dramatically influences their surface expression (Manganas and Trimmer, 2000), we co-transfected Kv1.1 subunits together with Kv1.2 or Kv1.4. For example, Kv1.1 transfected alone localizes poorly to the cell surface (Fig. 5A), but upon co-expression with Kv1.2 or Kv1.4, Kv1.1 can be detected uniformly at the cell surface using Kv1.1 ext antibodies (Figs. 5B and 5C). As for Kv1 channels, expression of PSD-95 or PSD-93 alone revealed a uniform distribution throughout the cell without any apparent clustering (Figs. 5D and 5E). However, as shown previously (Manganas et al., 2001), upon co-expression of PSD-95 with Kv1.1/Kv1.2 (Fig. 5F) or Kv1.1/Kv1.4 (Fig. 5G) the proteins redistributed into clearly defined surface clusters. Similarly, co-expression of PSD-93 with Kv1.1/Kv1.2 (Fig. 5H) or Kv1.1/Kv1.4 (Fig. 5I) also resulted in the formation of large surface clusters comprised of Kv1 channel subunits and PSD-93. Thus, as with the MAGUKs PSD-95 and SAP-97 (Kim and Sheng, 1996b), PSD-93 can interact with and cluster Kv1 channels at the cell surface.

PSD-93 is required for Kv1 channel localization at the AIS

Is PSD-93 required for clustering of Kv1 channels at the AIS? To answer this question we prepared H1 promoter driven short-hairpin RNA (shRNA) plasmids to silence expression of PSD-93 in cultured hippocampal neurons. We confirmed the efficacy of gene silencing by co-expressing PSD-93-EGFP and PSD-93 shRNA plasmids in COS7 cells (data not shown). We found two functional shRNA plasmids (called R1 and R3, see materials and methods for details) that targeted different sequences in PSD-93 and that dramatically reduced the amount of PSD-93-EGFP expressed in the transfected COS7 cells. We then co-transfected R1, R3, or a control shRNA expression plasmid together with an enhanced green fluorescent protein (EGFP) expression plasmid (to identify transfected neurons) into cultured rat hippocampal neurons. In the control shRNA transfected hippocampal neurons, PSD-93 was detected at the AIS of 49.4% (n=7; transfected cell numer=510) of EGFP+ neurons (Fig. 6A; arrowhead and Fig. 6C). However, only 7.3% (n=6; transfected cell number=521) and 1.6% (n=6; transfected cell number=474) of EGFP + cells had detectable endogenous AIS PSD-93 when transfected with the R1 and R3 shRNA expression plasmids, respectively (R3; Fig. 6A; arrowhead and Fig. 6C, R1; Fig. S1B). Non-transfected cells in the same culture dish had normal PSD-93 at the AIS (Fig. 6A; arrow).

We next determined whether knockdown of PSD-93 expression affects Kv1 channel localization at the AIS. When transfected with the control shRNA, we found that 43.5% (n=5; transfected cell number=603) of EGFP+ neurons had Kv1.4 immunoreactivity at the AIS (Fig. 6B; arrowhead and Fig 6C). The AIS Kv1.4 signal intensity of control shRNA transfected neurons was similar to that in non-transfected cells (Fig. 6B; arrow). In contrast, fewer neurons

transfected with the R1 or R3 shRNAs had Kv1.4 immunoreactivity at the AIS. Only 24.9% (n=6; transfected cell number=579) and 20.0% (n=6; transfected cell number=531) of EGFP + neurons had detectable Kv1.4 at the AIS when transfected with the R1 and R3 shRNA expression constructs, respectively (R3; Fig. 6B; arrowhead and Fig. 6C, R1; Fig. S1B), and similar results were seen for Kv1.1 (Fig. S1C). Because a few neurons still had weakly detectable Kv1.4 immunoreactivity at the AIS, we determined whether other MAGUKS compensate for loss of PSD-93. However, we did not detect PSD-95, SAP102 or SAP97 at the AIS of neurons lacking PSD-93 (Fig. S2), suggesting the residual weak Kv1.4 immunoreactivity reflects incomplete knockdown of PSD-93 and differences between anti-PSD-93 and anti-Kv1.4 sensitivities.

We next analyzed PSD-93 −/− mice to confirm the dependence of Kv1 channel AIS localiztion on its interaction with PSD-93. As with P10 rats, cortical neurons of wild-type (WT) P10 mice had abundant Kv1.2 channel immunoreactivity at distal segments of the AIS where it colocalized with βIV spectrin (Fig. 7A, arrow). In contrast, we never observed AIS Kv1.2 immunoreactivity in P10 PSD-93 −/− mice (Fig. 7B; arrow). Thus, PSD-93 is required for Kv1 channel localization at the AIS.

Caspr2 and TAG-1 are located at the AIS

Since Caspr2 and TAG-1 mediate clustering and localization of Kv1 channels at juxtaparanodes (Poliak et al., 1999; Poliak et al., 2003; Traka et al., 2003), we considered whether these CAMs might also be located at the AIS. In rat hippocampal neuron cultures we found Caspr2 and TAG-1 immunoreactivity at the AIS, and this immunoreactivity colocalized with Nav channels (Figs. 8A and 8C) and Kv1.2 (Fig. 8B). This observation agrees with a previous report by Inda et al. (2006) demonstrating Caspr2 at the AIS of human cortical neurons *in vivo*. Caspr2 also interacts with protein 4.1B at juxtaparanodes (Poliak et al., 2001; Denisenko-Nehrbass et al., 2003). However, we did not detect any enrichment of 4.1 proteins at the AIS (Figs. 8D and S3).

Caspr2 is not required for Kv1 channel localization at the AIS

Is Caspr2 required for Kv1 channel localization at the AIS? To answer this, we immunostained brains from Caspr2 −/− and +/− littermates. In both genotypes, we found Kv1.2 immunoreactivity that colocalized with βIV spectrin at the AIS of hippocampal (Fig. 9, arrows) and cortical (data not shown) neurons. Thus, Caspr2 is enriched at the AIS and interacts with Kv1 channels through PSD-93, but is not required for Kv1 channel localization at the AIS.

DISCUSSION

Ion channels at the AIS

Information processing in the nervous system depends on exquisitely regulated neuronal input and output. Neuronal input occurs in the form of synaptic transmission, and output occurs when these synaptic signals are integrated and depolarize the membrane sufficiently to elicit an action potential in the axon (Palmer and Stuart, 2006). The AIS functions as a physical bridge between the somatodendritic and axonal domains and as a master regulator linking information processing and transmission. Moreover, the excitability of the AIS depends on the types, density, and location of clustered ion channels. Nav channels initiate the action potential, and Nav1.1, Nav1.2, and Nav1.6 occur alone and in combinations at the AIS of different CNS neurons (Garrido et al., 2003; Van Wart et al., 2007) (Boiko et al., 2003; Van Wart et al., 2007). KCNQ2 and KCNQ3 Kv channels are also enriched at the AIS in an ankG dependent manner (Devaux et al., 2004; Pan et al., 2006). Mutations in KCNQ2 and KCNQ3 cause neonatal epilepsies, emphasizing their role in regulating neuronal excitability (Jentsch, 2000). In addition, Kv1.2 subunits occur at the AIS of diverse neurons (Inda et al., 2006; Kuba et al.,

2006; Van Wart et al., 2007). Dodson et al. (2002) showed Kv1.1 and Kv1.2 subunits are enriched at the AIS of medial nucleus of the trapezoid body (MNTB) neurons and regulate action potential firing. We extend these results to show that in hippocampal and cortical neurons Kv1.4 is also located at the AIS, where it likely forms heteromultimeric channels with Kv1.1, Kv1.2, and Kvβ2. Kv1 channel subunits are capable of heteromultimerizing promiscuously, and the addition of Kv1.4 subunits in the channel would be expected to convert Kv1.1/Kv1.2 subunit-containing channels from non-inactivating delayed rectifiers to channels with transient A-type currents. Regulating the Kv1 channel subunit composition at the AIS may modulate excitable properties of the AIS. Kole et al. (2007) recently showed that Kv1 channels directly modulate the action potential waveform in the AIS of layer 5 pyramidal neurons, and that this facilitates transmitter release at intracortical synapses. These ideas are consistent with the observation that Kv1.1 −/− mice have seizures throughout life (Smart et al., 1998) and a subset of patients with long-term epilepsy have autoantibodies against Kv1 channels (Majoie et al., 2006).

Mechanism of Ion Channel Clustering at the AIS

Nav and KCNQ2/3 channel localization depends on direct binding to the scaffold ankG (Garrido et al., 2003; Pan et al., 2006). Some data suggested that ankG localization to the AIS depends on interaction with the cytoskeletal protein βIV spectrin, (Komada and Soriano, 2002). However, Yang et al. (2007) recently demonstrated that like Nav and KCNQ2/3 channels, βIV spectrin localization to the AIS and nodes also depends on ankG, pointing to ankG's central role in the molecular organization of these excitable domains (Hedstrom et al., 2007). The mechanisms that initiate ankG clustering at the AIS remain unknown.

As for Nav and KCNQ2/3 channels, Kv1 channel localization to the AIS depends on a scaffolding protein, albeit through binding to PSD-93 rather than binding to ankG. It is interesting to note that although other MAGUKs such as SAP102 appear to compensate for loss of PSD-93 from some synapses (Elias et al., 2006), this is not true at the AIS. Hence, PSD-93 has a highly specific function to cluster Kv1 channels at the AIS.

How does PSD-93 become localized to the AIS? One possibility is through its interaction with one of the three CAMs previously described at the AIS: NrCAM, NF-186, and Caspr2. All three of these proteins have putative C-terminal PDZ binding motifs (-NSFV, -YSLA, and -EWLI, respectively; Sheng and Sala, 2001), and NrCAM, but not NF-186, binds to the MAGUK SAP102 (Davey et al., 2005). However, neither NF-186 nor NrCAM are recruited into large surface clusters when co-expressed with PSD-93 and Kv1 channels (unpublished results). Although Caspr2 is also found only at the distal AIS (Inda et al., 2006) and is required for Kv1 channel clustering at the juxtaparanode (Poliak et al., 2003; Traka et al., 2003), the results presented here show that Caspr2 is not required for Kv1 channel localization at the AIS since *Caspr2-null* mice have Kv1 channels at the AIS.

Among all the AIS proteins previously identified, only Kv1 channels and Nav1.6 are restricted to the distal region of the AIS. In contrast, in some neurons Nav1.1 is located in the proximal segment of the AIS (Van Wart et al., 2007; Ogiwara et al., 2007). This observation suggests that the different channel distributions between the proximal and distal AIS depend not only on ankG, but on other mechanisms as well. Based on its distal AIS localization, Kv1 channel targeting may be related to the localization of Nav1.6 or its associated proteins. Nav channels consist of both a pore-forming α subunit and an accessory β subunit. Four Navβ subunits have been identified and are members of the immunoglobulin superfamily of CAMs. Both Navβ1 and Navβ3 can interact with NF-186 and NrCAM (Ratcliffe et al., 2001), but these Navβ subunits do not interact with each other (McEwen and Isom, 2004). It is possible that the distinct channel distributions of Nav channel α-subunits may reflect differences in the interactions among Navβ subunits. Among the Navβ subunits, only Navβ4 has a PDZ binding motif like

Kv1 channel α subunits. Navβ4 is thought to interact with Nav1.6 and be responsible for 'open-channel block' of Nav channels, permitting high-frequency firing in neurons (Grieco et al., 2005). We speculate that PSD-93 may be localized through interactions with Navβ4, spatially and molecularly coupling Nav1.6 and Kv1 channels at the AIS. Alternatively, PSD-93 may modulate Navβ4's ability to mediate open-channel block by binding to the c-terminus of Navβ4.

A multi-step sequence of events for AIS clustering of Nav channels has been proposed (Fache et al., 2004). Both endocytic elimination from somatodendritic domains and directed sorting of channels to axons are thought to contribute to their localization (Garrido et al., 2001; Fache et al., 2004). Subsequently, Nav channels are selectively retained at the AIS through interactions with ankG. Recently, Gu et al. (2006) reported that axonal trafficking of Kv1 channels depends on interactions between accessory Kvβ2 subunits, a microtubule plus-end tracking protein called EB1, and the microtubule-based motor KIF3/kinesin II. Similarly, Rivera et al. (2007) provided evidence that KIF5B interacts with Kv1 channels and contributes to their axonal localization. Thus, as for Nav channels, it is likely that the AIS clustering of Kv1 channels is a multi-step sequence of events, including directed trafficking to the axon followed by retention of channels at the AIS by PSD-93. Although we have focused on their AIS localization, Kv1 channels can also be detected along unmyelinated axons and in somatodendritic domains depending on the neuron (Trimmer and Rhodes, 2003; Khavandgar et al., 2005). Thus, additional mechanisms regulating Kv1 channel localization in neurons remain to be identified.

In conclusion, PSD-93 functions not only at synapses, but also as a scaffold, independent of Caspr2, to cluster and restrict Kv1 channels at the AIS. These results support the idea that scaffolding proteins, rather than CAMs, direct the assembly of ion channel complexes at the AIS. Thus, although many protein components may be common to the AIS and juxtaparanodes, the mechanisms that mediate their assembly are distinct. Like nodes of Ranvier, the juxtaparanodes are organized from the 'outside-in' but the AIS is assembled from the 'inside-out' (Dzhashiashvili et al., 2007; Hedstrom et al., 2007; Yang et al., 2007).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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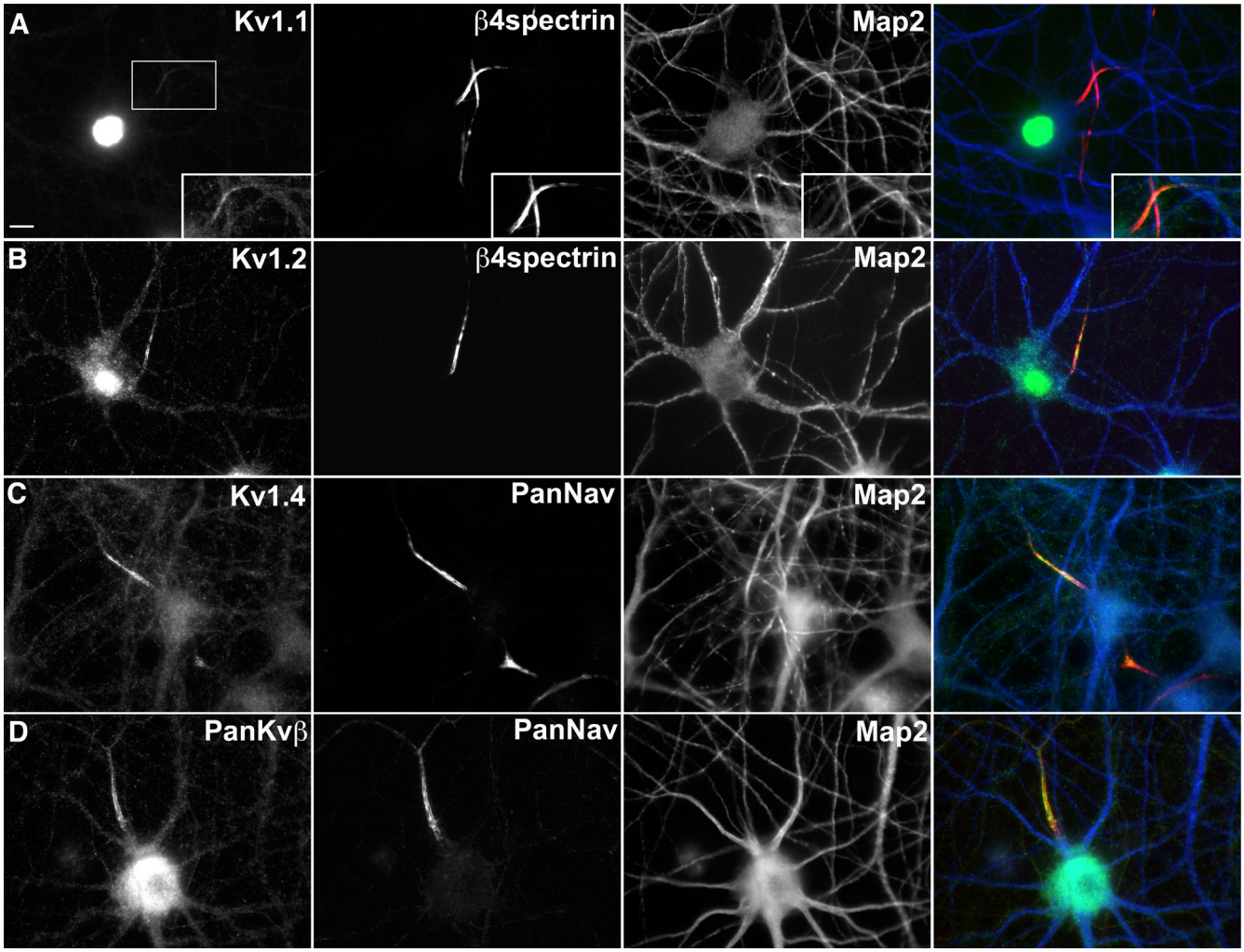

Figure 1.

Kv1 channel α- and β-subunits are enriched at the AIS in cultured hippocampal neurons. Kv1.1 (**A**), Kv1.2 (**B**), Kv1.4 (**C**), and Kvβ (**D**) subunits (green) are located at the AIS where they colocalize with βIV spectrin or Nav channels (red). Somatodendritic domains are defined by MAP2 immunoreactivity (blue). Scale bar: 10 μm.

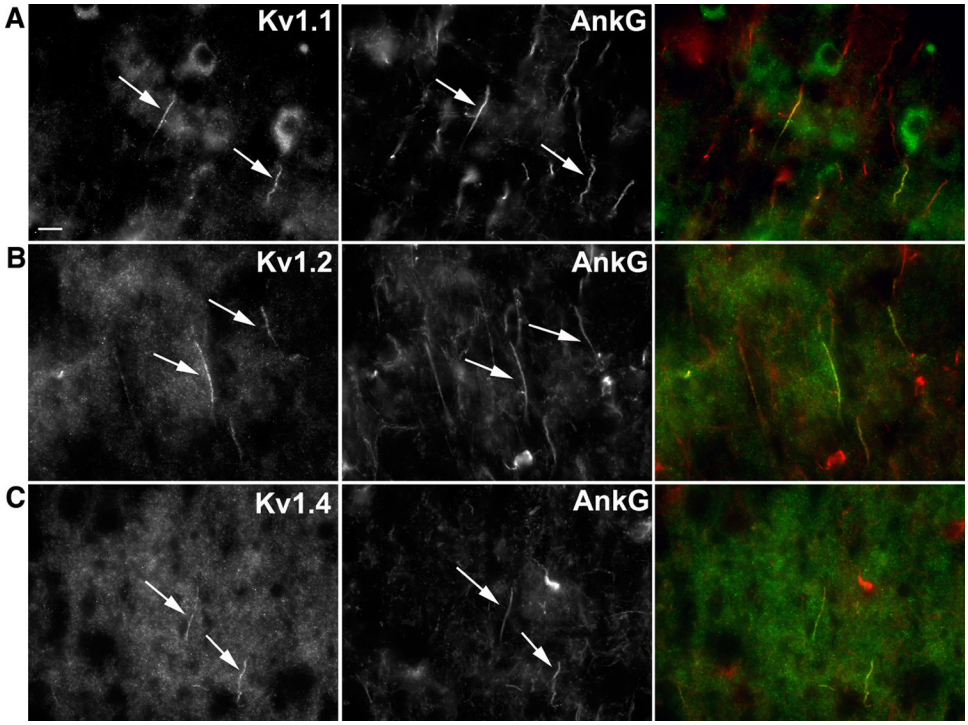

Figure 2.

Kv1 channel α-subunits are enriched at the AIS of P10 rat cortical neurons. Kv1.1 (**A**), Kv1.2 (**B**), and Kv1.4 (**C**) α-subunits (green) colocalize with ankG (red) at the AIS (arrows). Scale bar: 10 μm.

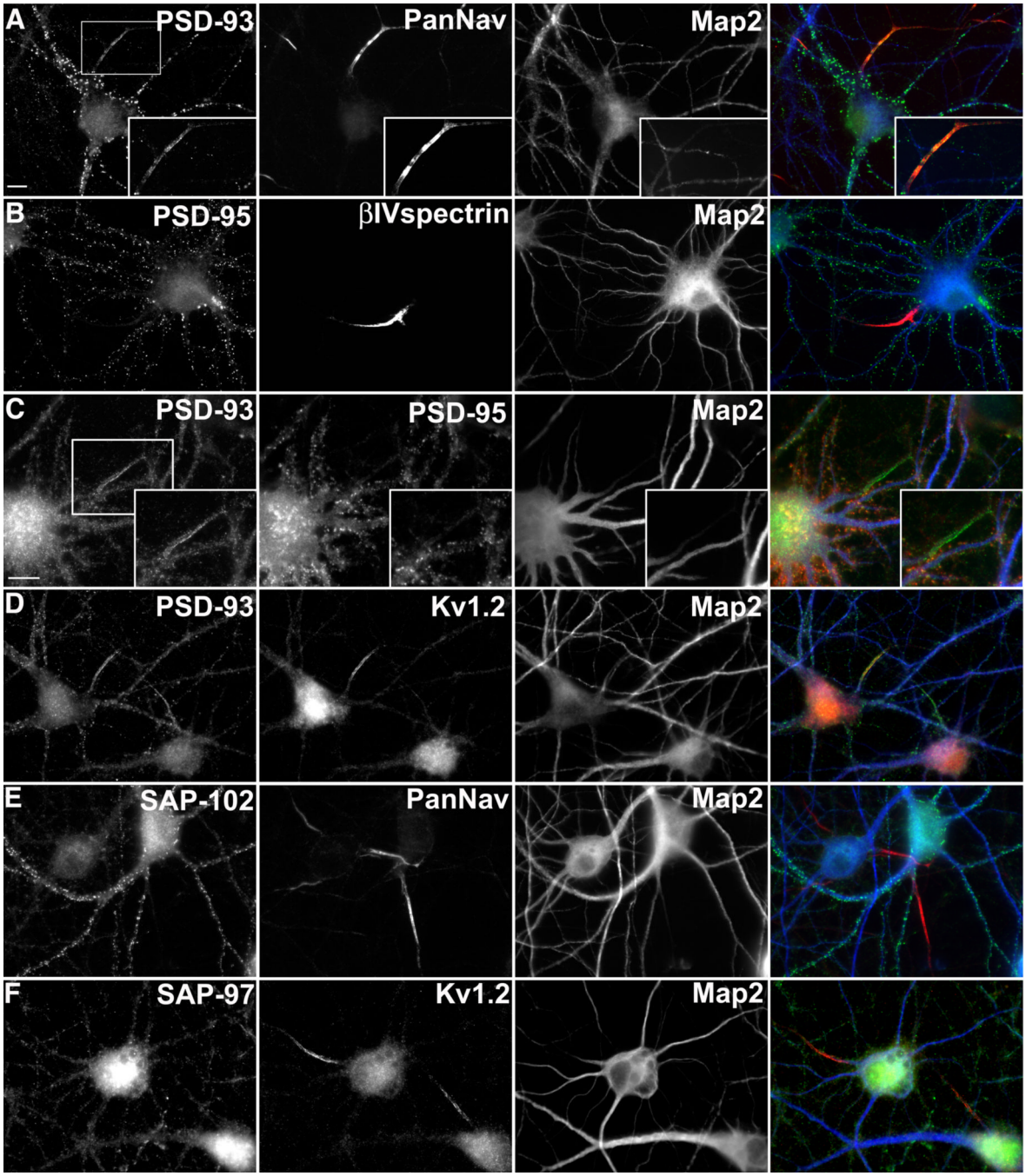

Figure 3.

PSD-93, but not PSD-95, is enriched at the AIS of cultured hippocampal neurons. **A**, PSD-93 (green) is located at synapses and the AIS (box and inset). At the AIS PSD-93 colocalizes with Pan-Nav immunoreactivity (red). **B**, PSD-95 (green) is located at synapses, not at βIV spectrin-labeled initial segments (red). **C**, Double-immunostaining for PSD-93 (green) and PSD-95 (red) shows PSD-93, but not PSD-95, at the AIS (inset). **D**, PSD-93 (green) and Kv1.2 (red) immunoreactivity colocalizes at the AIS of cultured hippocampal neurons. **E**, SAP102 (green) and **F**, SAP97 (green) immunoreactivity are located at synapses along dendrites, but do not colocalize with Nav channels (red) at the AIS. Somatodendritic domains are defined by MAP2 immunostaining (blue). Scale bars: 10 μm.

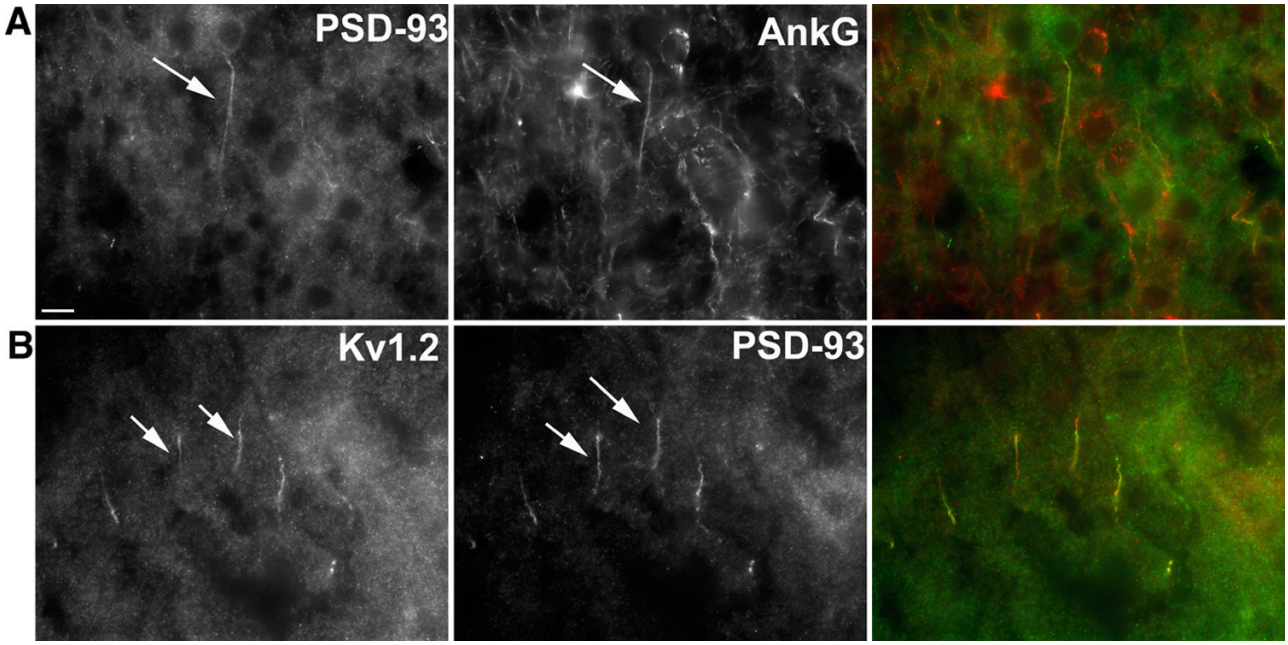

Figure 4.

PSD-93 is enriched at the AIS of P10 rat cortical neurons. **A**, PSD-93 (green) colocalizes with ankG (red) immunostaining (arrows). **B**, Kv1.2 α-subunits (green) colocalize with PSD-93 (red) at the AIS (arrows). Scale bar: 10 μm.

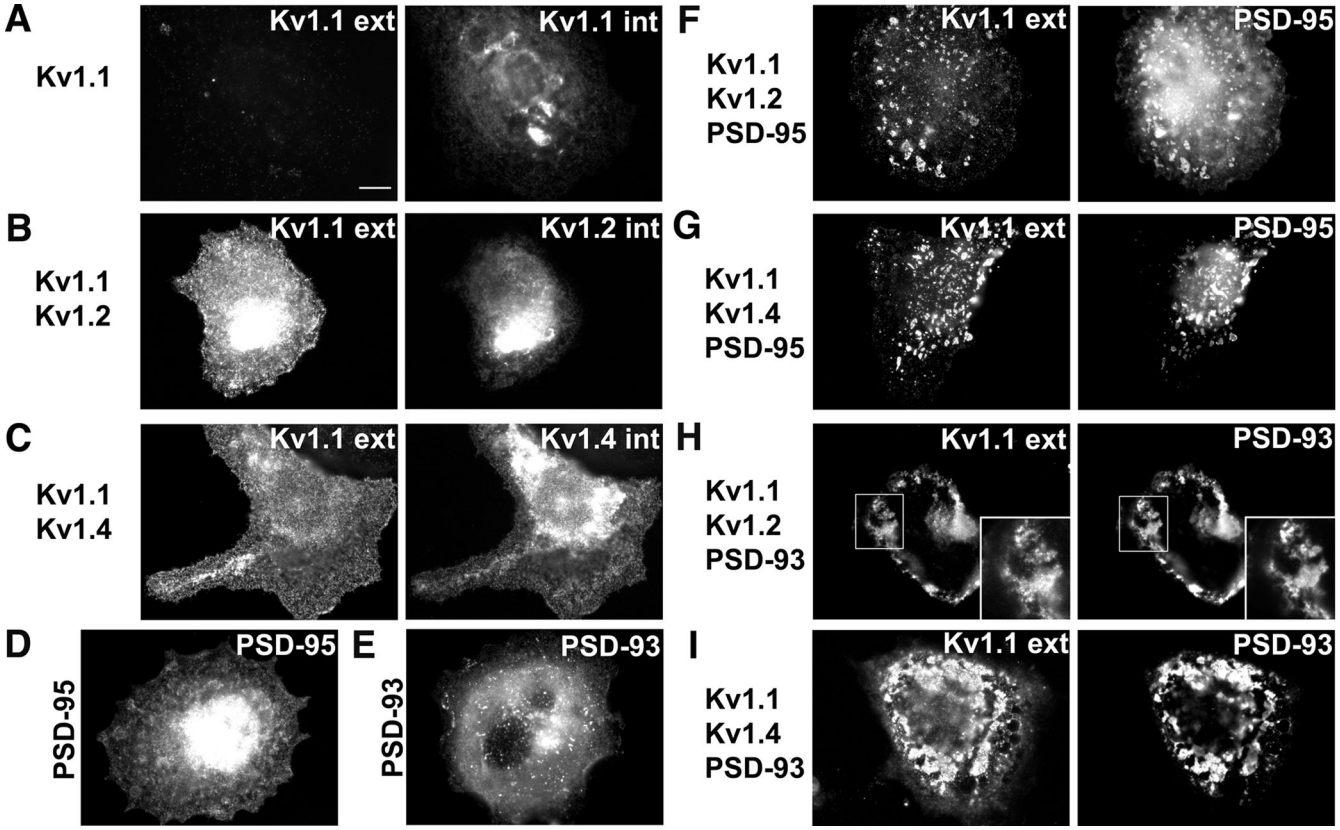

Figure 5.

Co-expression of Kv1 channel α-subunits with PSD-93 results in the formation of large surface clusters of Kv1 channels in COS7 cells. The cDNAs used in the transfections are listed at the left of each set of images. **A**, Kv1.1 transfected cells labeled with antibodies against extracellular (Kv1.1 ext) or intracellular (Kv1.1 int) epitopes. **B**, **C**, Co-expression of Kv1.1 with Kv1.2 or Kv1.4 α-subunits promotes the uniform surface expression of Kv1.1. **D**, **E**, PSD-95 or PSD-93 transfected COS7 cells reveals a uniform cytoplasmic staining for the introduced MAGUK. **F**, **G**, Co-expression of Kv1.1 with Kv1.2 (**F**) or Kv1.4 (**G**) and PSD-95 results in the formation of large Kv1 channel surface clusters. Kv1.1 was detected using the Kv1.1ext antibody. **H**, **I**, Co-expression of Kv1.1 with Kv1.2 (**H**) or Kv1.4 (**I**) and PSD-93 results in the formation of large Kv1 channel surface clusters. Kv1.1 was detected using the Kv1.1ext antibody. Scale bar: 10 μm.

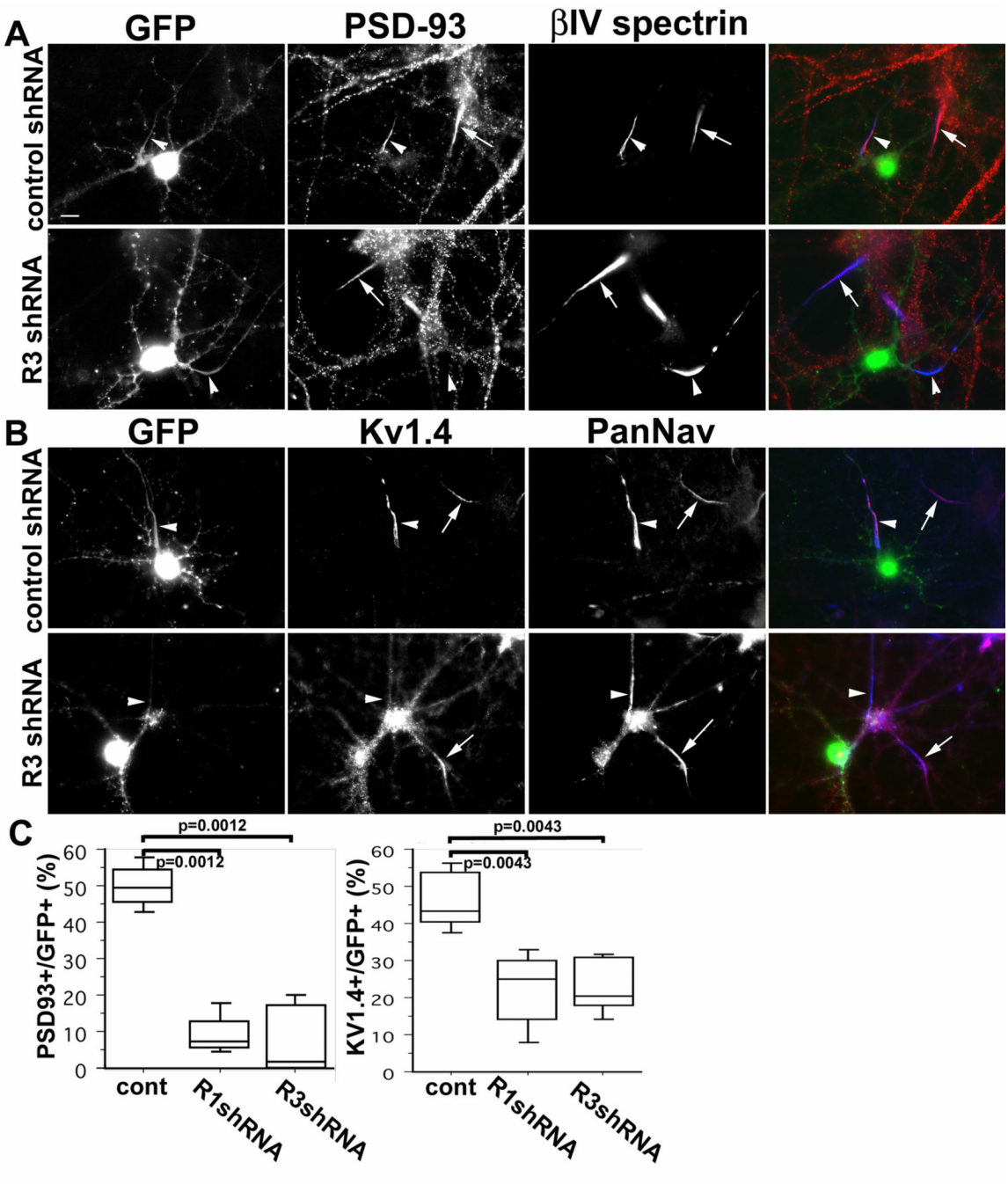

Figure 6.

Silencing of PSD-93 by shRNA expression plasmids disrupts Kv1 channel clustering at the AIS. Cultured hippocampal neurons were transfected with shRNA expression vectors (R3) targeting PSD-93. Transfected neurons are identified by GFP signal. Initial segments are identified by βIV spectrin (**A**) or Pan Nav (**B**) immunostaining. Initial segments of transfected and untransfected neurons are indicated by arrowheads and arrows, respectively. **A**, **B**, Control shRNA expression plasmid transfected into hippocampal neurons has no effect on PSD-93 (**A**) or Kv1.4 (**B**) clustering at the AIS (arrowheads). **A**, Transfection with R3 shRNA expression plasmids eliminates immunostaining for PSD-93 at the AIS (arrowheads) of GFP + cells, but PSD-93 immunoreactivity can be detected in untransfected GFP- neurons. **B**,

Transfection with R3 shRNA expression plasmids eliminates, immunostaining for Kv1.4 at the AIS (arrowheads) of GFP+ cells, but Kv1.4 immunoreactivity can be detected in untransfected GFP- neurons. Scale bar: 10 μm.

C, Quantitative analysis for the number of GFP+ cells with PSD-93 or Kv1.4 immunoreactivity at the AIS. Boxes, 75th percentile with the median indicated; bars, 10th and 90th percentiles. Analyzed using the Mann-Whitney *u* test.

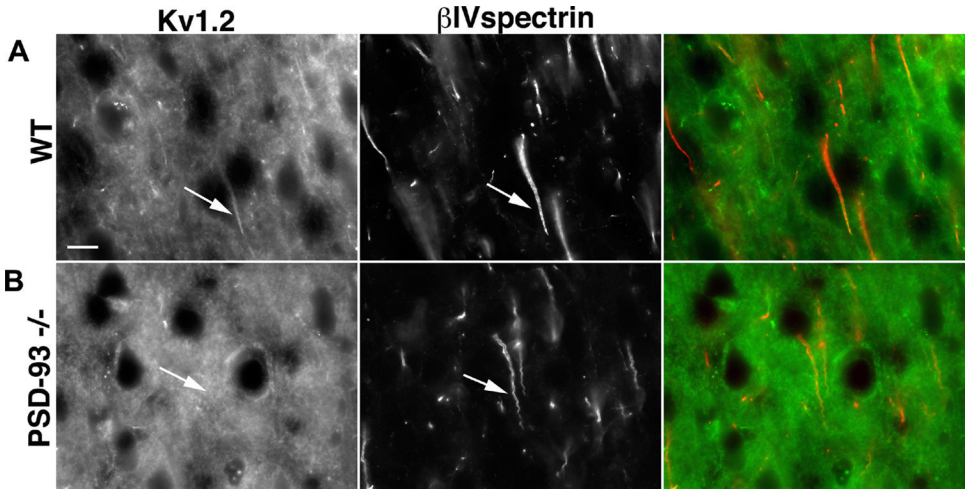

Figure 7.

Kv1 channels are not clustered at the AIS of P10 cortical neurons in PSD-93 −/− mice. **A**, Kv1.2 (green) colocalizes with AIS βIV spectrin (red) immunostaining (arrows) in P10 wild-type (WT) mouse cortex. **B**, Kv1.2 (green) is not enriched at βIV spectrin –labeled AIS (arrow) in P10 PSD-93 −/− mice. Scale bar: 10 μm.

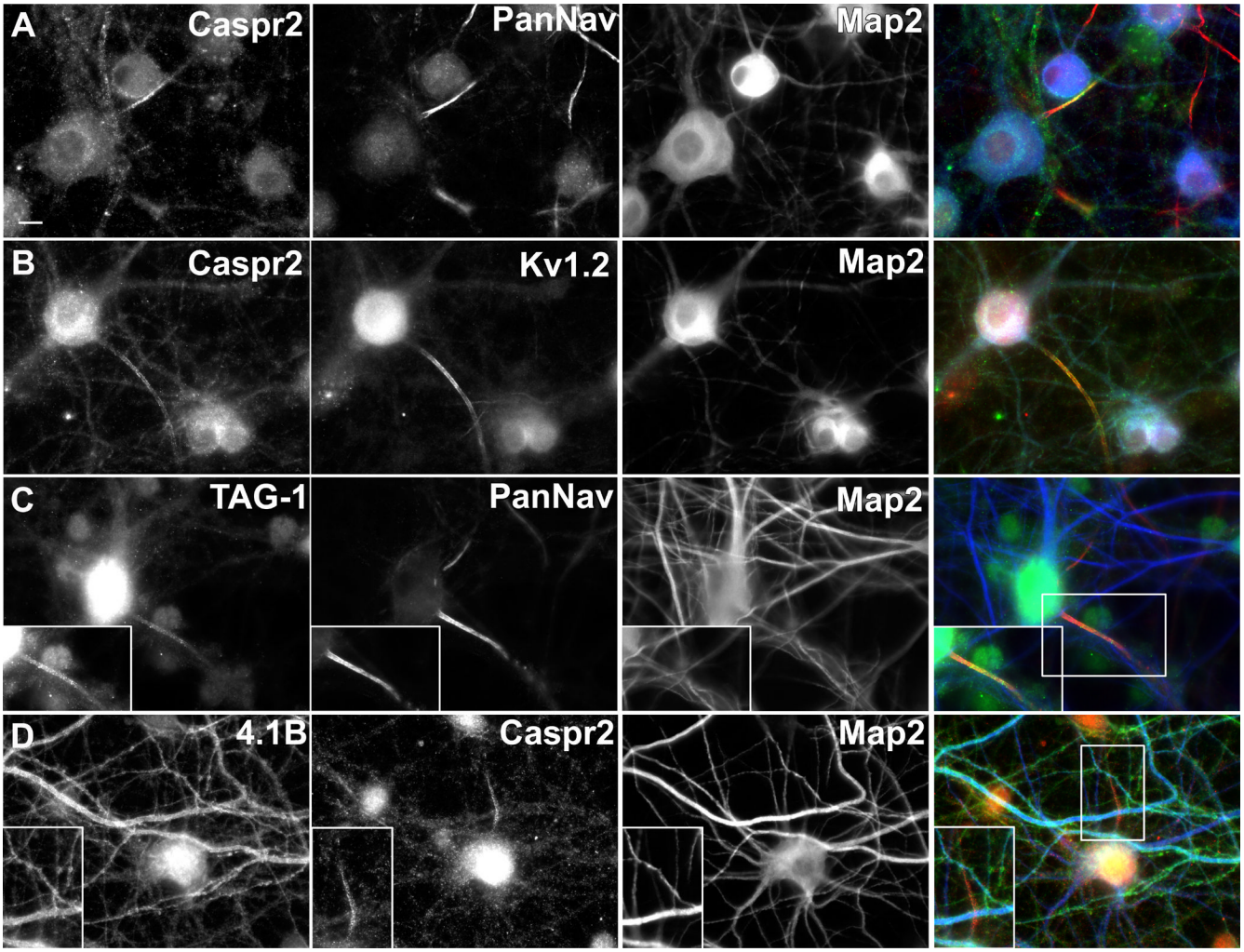

Figure 8.

Caspr2 and TAG-1 are located at the AIS of cultured hippocampal neurons. **A**, Caspr2 immunostaining (green) colocalizes with PanNav immunoreactivity (red) at the AIS. **B**, Caspr2 (green) and Kv1.2 (red) colocalize in cultured hippocampal neurons. **C**, **TAG-1** (green) and PanNav (red) immunoreactivity colocalize at the AIS of cultured hippocampal neurons. Note, the inset image shows the AIS with increased brightness in the TAG-1 channel. **D**, Protein 4.1B (green) does not colocalize with Caspr2 (red) at the AIS of cultured hippocampal neurons. Somatodendritic domains are defined by MAP2 staining (blue). Scale bar: 10 μm.

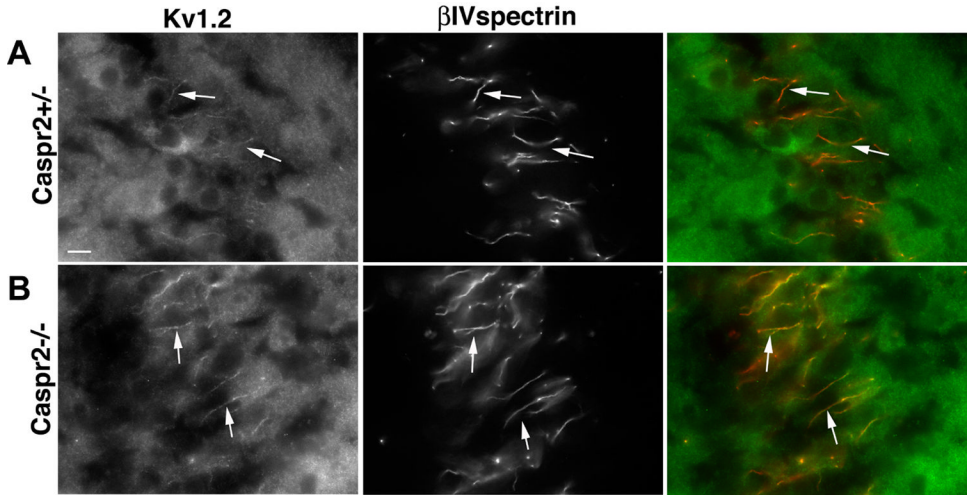

Figure 9.

Kv1 channels are clustered at the AIS of P10 Caspr2 −/+ (**A**) and Caspr2 −/− (**B**) hippocampus (arrows). Kv1.2 (green) colocalizes with βIV spectrin (red) at the AIS (arrows). Scale bar: 10 μm.