Introduction

Saltatory electric conduction requires clustered voltage-gated sodium channels (VGSCs) at axon initial segments (AIS) and nodes of Ranvier (NR). A dense membrane undercoat is present at these sites, which is thought to be key for the focal accumulation of channels. Here, we prove that βIVΣ1 spectrin, the only βIV spectrin with an actin-binding domain, is an essential component of this coat. Specifically, βIVΣ1 coexists with βIVΣ6 at both AIS and NR, being the predominant spectrin at AIS. Removal of βIVΣ1 alone causes the disappearance of the nodal coat, an increased diameter of the NR, and the presence of dilations filled with organelles. Moreover, in myelinated cochlear afferent fibers, VGSC and ankyrin G clusters appear fragmented. These ultrastructural changes can explain the motor and auditory neuropathies present in βIVΣ1 −/− mice and point to the βIVΣ1 spectrin isoform as a master-stabilizing factor of AIS/NR membranes.

βIVΣ1 spectrin stabilizes the nodes of Ranvier and axon initial segments

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Saltatory electric conduction requires clustered voltage-gated sodium channels (VGSCs) at axon initial segments (AIS) and nodes of Ranvier (NR). A dense membrane undercoat is present at these sites, which is thought to be key for the focal accumulation of channels. Here, we prove that βIVΣ1 spectrin, the only βIV spectrin with an actin-binding domain, is an essential component of this coat. Specifically, βIVΣ1 coexists with βIVΣ6 at both AIS and NR, being the predominant spectrin at AIS. Removal of βIVΣ1 alone causes the disappearance of the nodal coat, an increased diameter of the NR, and the presence of dilations filled with organelles. Moreover, in myelinated cochlear afferent fibers, VGSC and ankyrin G clusters appear fragmented. These ultrastructural changes can explain the motor and auditory neuropathies present in βIVΣ1 −/− mice and point to the βIVΣ1 spectrin isoform as a master-stabilizing factor of AIS/NR membranes.

Introduction

Saltatory electric conduction in myelinated neurons relies on the compartmentalized distribution of ion channels along the axons. Voltage-gated sodium channels (VGSCs), in particular, are clustered at axon initial segments (AIS) and nodes of Ranvier (NR), whereas potassium channels reside in the juxtaparanodes, which are separated from the NR by the interposition of the paranodes (Poliak and Peles, 2003; Salzer, 2003). Opening of VGSCs at AIS and NR triggers the generation and regeneration of action potentials, respectively. Changes in the expression and/or localization of proteins enriched at AIS/NR, paranodes, and juxtaparanodes affect nerve conduction and cause neuropathies (Suter and Scherer, 2003).

Since their first description by electron microscopy (Rob- erson, 1957), it has been appreciated that NR display distinctive structural properties, including a thick coat beneath the plasma membrane, which most likely reflects the assembly of cytoskeletal proteins involved in the clustering of VGSCs. Recent studies have shown that anchoring of sodium channels to the cortical actin cytoskeleton is mediated by the binding of their cytoplasmic tail to ankyrins and spectrins. Originally discovered in erythrocytes, ankyrins (Bennett and Stenbuck, 1979) and spectrins (Marchesi and Steers, 1968) are now clearly appreciated as key organizers and scaffolding components of membrane microdomains in virtually all cells, including neurons (Bennett and Baines, 2001). Increasing evidence points to spectrins as also having a role in membrane trafficking (De Matteis and Morrow, 2000).

βIV spectrin was recently identified as the specific spectrin found at AIS and NR (Berghs et al., 2000), where it interacts with VGSCs through its binding to ankyrin G (Jenkins and Bennett, 2001; Komada and Soriano, 2002). Alternative splicing generates six βIV spectrin isoforms (βIVΣ1–βIVΣ6; see Fig. 1 A; Berghs et al., 2000; Tse et al., 2001, Komada and Soriano, 2002). Genetic removal of multiple βIV spectrins in mice reduces immunoreactivity for ankyrin G and VGSCs at AIS and NR (Komada and Soriano, 2002). These mice display tremors, clasping of the hind limbs, and altered gait, consistent with a decreased nerve conductivity. A similar phenotype is present in the various
“quivering” mouse strains, each arising from spontaneous mutations in the βIV spectrin gene causing progressively longer truncations of βIV spectrins (Parkinson et al., 2001). Preliminary evidence suggested that βIV1 and a 140-kD βIV spectrin (Berghs et al., 2000, 2001), conceivably βIV6 (Komada and Soriano, 2002), are found at AIS and NR, whereas βIV5 is in the nucleus (Tse et al., 2001). Accordingly, it is still unclear how many βIV spectrin isoforms are found at AIS and NR. Identification of these isoforms and of the isoforms whose absence is responsible for the phenotypes in βIV spectrin-deficient mice may help to elucidate which interactions of spectrin are most critical for its function at AIS and NR.

Here, we prove that βIV1 and βIV6 are present at both central and peripheral AIS and NR and that absence of βIV1 alone destabilizes AIS/NR generating a quivering phenotype. Moreover, our data highlight the physiological relevance of βIV1 in the myelinated afferent fibers of the auditory pathway, which innervate the sensory hair cells.

Results and discussion

Generation of βIV1 spectrin –/– mice

Exon 2 of mouse βIV spectrin, which encodes the first methionine, was removed by homologous recombination (Fig. 1 B). PCR analyses confirmed the targeting of βIV spectrin in heterozygous (+/−) and homozygous mutant (−/−) mice (Fig. 1 C). βIV1 –/− mice were born with Mendelian frequency, but were smaller than +/+ and +/− littermates (weight at 3 wk: +/+, 13.2 ± 0.8 g, n = 5; +/-, 12.4 ± 0.7 g, n = 21, P < 0.01; −/−, 10.0 ± 0.6 g, n = 4; P < 0.05) and displayed tremors, mild dysmetria, clumsy gait, and dragging of the hind limbs. Despite these deficits, 3- and 5-mo-old βIV1 −/− mice scored like +/+ mice.
for their running wheel activity for 1 h (unpublished data) or open field locomotion for 20 min (distance covered, number of rearing, and time at rest; unpublished data). Their lifespan was normal and no abnormalities were found by pathological survey of multiple organs, including the brain (unpublished data).

Immunoblot with two antibodies confirmed the deletion of βIVΣ1. The NT antibody binds an NH$_2$-terminal epitope in βIVΣ1, βIVΣ2, and βIVΣ4, whereas the specific domain (SD) antibody binds the domain unique to βIVΣ1, βIVΣ3, and βIVΣ6 (Fig. 1 A). In +/+ mouse brain, the NT antibody detected a single protein of 250 kD (Fig. 1 D), corresponding to βIVΣ1 (Berghs et al., 2000). This reactivity was absent in βIVΣ1 −/− mice and was abolished by preincubating the antibody with its immunogenic peptide. Notably, the NT antibody did not detect other isoforms besides βIVΣ1. Thus, the expression in vivo of βIVΣ2 and βIVΣ4 remains to be proven. The SD antibody also did not detect βIVΣ1 in −/− mice, while its reactivity with βIVΣ6 was unaffected (Fig. 1 E). As in rat (Berghs et al., 2000), βIVΣ1 was already present in mouse embryonic brain, whereas βIVΣ6 only appeared after birth (Fig. 1 G). Notably, in the absence of βIVΣ1 there was no temporal or quantitative compensatory expression of βIVΣ6.

Coexistence of βIVΣ1 and βIVΣ6 at AIS and NR, with predominance of βIVΣ1 at AIS

The expression of βIVΣ1 during development suggested that this isoform plays a role at AIS, which are formed before NR. The postnatal appearance of βIVΣ6 parallels myelination and the progressive creation of NR. Presence of βIVΣ1 and βIVΣ6 in sciatic nerves, which include NR but no AIS, indicated that both isoforms are at NR (Fig. 1 F).

The NT antibody, which only binds βIVΣ1, stained AIS (Fig. 2, A and C, arrowheads) and NR (Fig. 2, A and C, arrows) in the cerebellum of +/+ mice (Fig. 2, A and C), but not in −/− mice (Fig. 2, B and D). This staining was blocked by the antigenic peptide and was not detected with the secondary antibody alone (unpublished data). These data prove that βIVΣ1 is found in both AIS and NR. The SD antibody labeled more intensively AIS and NR of +/+ mice than the NT antibody (Fig. 2 E), partly because it recognizes also βIVΣ6. In βIVΣ1 −/− mice, the SD labeling was reduced (Fig. 2, F and H) but not abolished, indicating that βIVΣ6 coexists with βIVΣ1 at AIS and NR. This labeling was more strongly reduced at AIS (Fig. 2, G and H, arrowheads) than at NR (Fig. 2 G and H, arrows), suggesting that βIVΣ1 is the major spectrin at AIS. This hypothesis was corroborated by evidence that in βIVΣ1 +/+ mice AIS were already visible at postnatal day 1 (P1; Fig. 2 I), whereas

![Figure 2](image-url)
in βIVΣ1 −/− mice, which still express βIVΣ6, they were only detected at P10 (Fig. 2 L), and more weakly than in +/+ mice (Fig. 2, L vs. K). As βIVΣ6 was still found at AIS and NR in the absence of βIVΣ1, whereas βIV spectrins were absent in AIS of Purkinje cells lacking ankyrin G (Jenkins and Bennett, 2001), it is conceivable that nodal targeting of βIV spectrins depends on their binding to ankyrin G.

**Altered shape of NR in βIVΣ1 −/− mice**

Next, we examined if βIVΣ1 is required for the integrity of central nervous system (CNS) and peripheral nervous system (PNS) nodes, as well as for the distribution of various markers in this compartment. Labeling of the cerebellum with SD (Fig. 3, A–D), anti-ankyrin G (Fig. 3, E–H), or anti-VGSCs (Fig. 3, I and J) antibodies showed that nodal width and length were increased in βIVΣ1 −/− mice. **Figure 3. Distribution of nodal markers in the CNS and PNS of βIVΣ1 +/+ and −/− mice. (A–J) Cerebellar sections of βIVΣ1 +/+ and −/− mice were double labeled as indicated for the following proteins: βIV spectrin (βIV sp.; SD antibody), Kv1.1, caspr/paranodin (caspr), ankyrin G (ankG), Kv1.2, VGSCs, and caspr2. The arrows in A–D, G, and H point to NR in axons of comparable size.**

![Image](986 The Journal of Cell Biology | Volume 166, Number 7, 2004)
brighter and thicker in βIVΣ1 −/− (Fig. 3, arrows in F vs. E). Because by immunoblot the levels of ankyrin G and VGSCs were not changed (unpublished data), the increased immunoreactivity may result from an enhanced accessibility of these antigens to antibodies in the absence of βIVΣ1. According to this interpretation, βIVΣ1, similar to βIVΣ6 (Komada and Soriano, 2002), binds ankyrin G through its repeat 15 and thereby to VGSCs.

Changes were also found in the paranodes and juxtaparanodes of βIVΣ1 −/− mice. The labeling for the paranodal marker caspr/paranodin was equally intense in βIVΣ1 −/− mice as in +/+ mice, but the length of the paranodes was reduced (Fig. 3, D vs. C, arrowheads). In βIVΣ1 −/− mice, the staining for potassium channels Kv1.1 (Fig. 3, B vs. A) and Kv1.2 (Fig. 3, H vs. G), which are normally restricted to the juxtaparanodes, was decreased and more spread, albeit the total reactivity for Kv1.1 was similar (average optical density: βIVΣ1 +/+, 0.242 ± 0.0002 vs. βIVΣ1 −/−, 0.244 ± 0.0004). Occasionally, Kv1.2 immunoreactivity invaded the paranodes (Fig. 3 H, arrowhead). Similar observations were made in the optic nerve (unpublished data). An altered distribution of Kv channels has also been observed in the quivering qvlnd mouse (Parkinson et al., 2001). As βIV spectrin is not detected at paranodes and juxtaparanodes, such changes can indirectly result from the alterations at the NR.

The NR in the sciatic nerves of βIVΣ1 −/− mice were shorter, larger, and geometrically less regular than in +/+ mice (Fig. 3, N–U). Measurement of 22 NR/genotype confirmed that in βIVΣ1 −/− mice their width was increased (P < 0.01), while their length was reduced (P < 0.05; Fig. 3 M). Absence of βIVΣ1 caused also alterations of the PNS paranodes and juxtaparanodes. The caspr labeling was less compact and could invade the NR (Fig. 3, U vs. T). Likewise, Kv1.1 and Kv1.2 were less clustered at juxtaparanodes (Fig. 3, Q vs. P) and could invade the paranodes (Fig. 3, O vs. N, asterisk). However, by immunoblot the levels of VGSCs and Kv1.2 were not changed (unpublished data). Thus, also in the PNS the lack of βIVΣ1 changed the pattern of nodal and juxtaparanodal markers.

Ultrastructural abnormalities of NR in βIVΣ1 −/− mice

To conclusively prove the occurrence of structural alterations in the absence of βIVΣ1, NR in the optic (CNS) and sciatic (PNS) nerves of βIVΣ1 +/+ and −/− mice were examined by electron microscopy. NR in the optic nerve of −/− mice were longer and swollen (Fig. 4, C vs. A), whereas
the electron dense membrane undercoat was absent (Fig. 4, C vs. A, arrows; and D vs. B). Ultrastructural changes were also observed in peripheral NR of \( \beta IV\Sigma 1 \sim / \sim / \sim \) mice (Fig. 4, G–K). Their length was reduced (Fig. 4, G vs. E), whereas lateral protrusions, which were never detected in \(+ / + / \sim \) mice, were found in \( 7 / 15 \) NR (Fig. 4, G and I–K, arrowheads). Large evaginations often contained vesicles (Fig. 4, I–K), some with an electron-dense core. Except within these dilations, the NR of \( \beta IV\Sigma 1 \sim / \sim / \sim \) mice contained fewer vesicles. Normally there is a higher density of vesicles at NR than at internodal regions, possibly because the nodal restriction slows axonal transport (Zimmermann, 1996). Consistently, vesicle density may be reduced in \( \beta IV\Sigma 1 \sim / \sim / \sim \) mice because NR are enlarged (see the following paragraph).

The membrane coat was also reduced in the NR of sciatic nerves (Fig. 4, H vs. F). As axons vary in diameter, the ratio between the diameter of each NR and their corresponding juxtaparanodes was calculated. This analysis showed that the diameter of NR in \( \beta IV\Sigma 1 \sim / \sim / \sim \) mice was \( 58 \pm 4\% \) of the corresponding juxtaparanode diameter, compared with \( 39 \pm 2\% \) in \(+ / + / \sim \) mice \( (P \leq 0.001; \) Fig. 3 L). This increase of \( \sim 20\% \) is consistent with the enlargement of \( \sim 25\% \) assessed on confocal images. Conversely, axoglial junctions at the paranodes of \( \beta IV\Sigma 1 \sim / \sim / \sim \) mice appeared normal (unpub-
lished data). Notably, even if βIVΣ6 is expressed at higher levels than βIVΣ1, it is not sufficient to stabilize the structure of AIS and NR, conceivably because βIVΣ6, unlike βIVΣ1, lacks the NH₂-terminal actin/protein 4.1 binding domain and is therefore less suited to anchor ankyrin G and surface proteins to the cortical cytoskeleton.

Auditory neuropathies in βIVΣ1 →−/− mice

The quivering phenotype and the alterations at AIS and NR suggested that nerve conduction is affected in βIVΣ1 →−/− mice. As auditory neuropathies are part of the quivering phenotype, auditory brainstem responses (ABR), were measured. ABR allow the assessment of hearing acuity by measuring the brain waves generated along the auditory pathway in response to acoustic stimuli. Unlike spontaneous quivering mice (Parkinson et al., 2001), βIVΣ1 →−/− mice displayed ABR (Fig. 5 A), but the latency of their auditory evoked potentials was increased (Fig. 5 C). Raising the stimulation rate further increased these latencies (Fig. 5 B), indicating an auditory neural fatigue. This deficit can reflect a delayed generation of action potentials by cochlear spiral ganglion neurons and an impaired conduction along the auditory pathway. A deficit of sensory hair cells can be excluded, as auditory thresholds were not changed (Fig. 5 D). This interpretation is consistent with the enrichment of βIVΣ1 at sites where the afferent fibers of the spiral ganglion sensory neurons leave the organ of Corti and become myelinated (Fig. 5, E and G, arrows). Therefore, these sites, where action potentials are thought to originate, can functionally equal the AIS in multipolar neurons. Likewise, the sites where βIVΣ1 clusters along myelinated afferent fibers (Fig. 5, E and F, arrowheads) can be equaled to the NR.

In βIVΣ1 →−/− mice, stainings for βIV spectrin (Fig. 5, I vs. H and insets I vs. H), VGS Cs (Fig. 5, K vs. j), and ankyrin G (not depicted) at both these sites were reduced and fragmented, whereas Kv1.2 was more spread (Fig. 5, K vs. J). Notably, each AIS-equivalent site was still positive for βIV spectrin, albeit the residual staining was altered. These data prove that βIVΣ1 and βIVΣ6 coexist at each AIS and that βIVΣ1 plays a key role in stabilizing the AIS structure. Our analyses extend those performed previously (Parkinson et al., 2001), as βIVΣ1 →−/− mice not only display prolonged latencies of ABR, but also suffer from auditory fatigue, which is a hallmark of neural hearing impairment (Hood, 1950). In general, the altered electrical conduction and axonal membrane trafficking resulting from the molecular and ultrastructural changes reported here may both contribute to the quivering features of βIVΣ1 →−/− mice, including ataxic tremor and auditory neuropathy.

In conclusion, our data provide a definitive molecular correlate to the membrane undercoat at NR. Specifically, they prove that βIVΣ1 is a major component of the nodal cortical coat and that in its absence the plastic properties of the nodal membrane are impaired. The scaffolding role of spectrins is known from the seminal studies in erythrocytes (Marchesi, 1985; Bennett and Baines, 2001), yet no formal proof for any spectrin isoform having this role in neuron has been provided before. This knowledge may open new opportunities for the understanding of axonal physiology in normal and pathological conditions.

Materials and methods

Generation of βIVΣ1 spectrin −/− mice

Plasmids from a C57BL mouse BAC library (Genome Systems Inc.) were isolated by screenings with a mouse βIV spectrin cDNA probe and digested with multiple restriction enzymes. Two fragments of 5.6 and 8.4 kb including exon 2 of βIV spectrin were used to generate a construct, in which a 600-bp fragment containing exon 2 was replaced with Pmc1-neo gene in the opposite orientation. The construct included a 5′-genomic insert of 3 kb preceded by thymidine kinase and a 3′-genomic insert of 4.2 kb. The vector linearized with Xhol was electroporated into C57BL/6 ES cells. Homologous recombination in selected clones was confirmed by Southern blot with a 320-bp EcoRI-BamHI probe. βIV spectrin +/− ES cells were microinjected into C57BL/6 blastocysts to generate βIV spectrin +/−/− mice, and then βIV spectrin +/−/− mice by intercrossing. Mice were genotyped by PCR with primers for exon 2 of mouse βIV spectrin (forward: 5′-ATGATGGGATCATTGTTTTTCTCTTG; reverse: 5′-TGTACGCGGAGAACACTCAAA and neo (forward: 5′-CCGGCCCTCAAGGCTCCAGTATG). Antibodies

The NT rabbit antibody was raised against residues 15–38 in exon 2 of human βIV spectrin. Specificity of the affinity-purified antibody was tested by immunoblot and immunocytochemistry on brain with or without a 5,000 molar excess of the antigenic peptide. The following antibodies were used: rabbit anti-βIV spectrin-SD (Berghs et al., 2001), anti-Kv1.2, and anti-caspar/paranodin (gifts of J.S. Trimmer [State University of New York, Stony Brook, NY] and J.-A. Girault [Institut du Fer à Moulin, Paris, France, respectively]), mouse anti-Kv1.1 (a gift of J.S. Trimmer), anti-pan-VGSC K5B35 (Randash et al., 1999), and anti-casp3/paranodin (Randash and Trimmer, 2001). The following antibodies were purchased: mouse anti-ankyrin G (Zymed Laboratories), anti-β6- and γ-tubulin and anti-neurofilament 200 (Sigma-Aldrich), and goat anti-rabbit and anti-mouse IgGs conjugated to Alexa 488 or 568 (Molecular Probes).

Immunoblot

Pregnant females, newborn, and adult mice were killed according to the German Animal Welfare Law. Brain tissues from E15 and E19 mouse embryos, 1- or 10-d-old mice, or adult mice were processed as described previously (Berghs et al., 2001). 50–150 μg of protein were separated by 6% SDS/PAGE and immunoblotted with primary antibodies (SD, 1:500; NT, 1:500; β-tubulin, 1:10000; γ-tubulin, 1:5000), followed by peroxidase-conjugated goat anti-rabbit or anti-mouse IgGs (Sigma-Aldrich; 1:5000). Signals were detected by ECL (Super Signal; Pierce Chemical Co.) with a LAS-3000 Bioimaging System (Fuji). Protein concentration was determined with the BCA assay (Pierce Chemical Co.).

Immunostaining

Anesthetized adult mice were transcardially perfused with 120 mM sodium phosphate buffer, pH 7.4, followed by 1% PFA in the same buffer at RT. Brain, optic nerves, and sciatic nerves were collected, post-fixed for an additional 1–3 h at RT, infiltrated with 30% sucrose in 120 mM sodium phosphate buffer, and frozen before cryosectioning. The labeling on 12-μm cryostat sections was performed as described previously (De Camilli et al., 1983) with anti-SD (1:50), NT (1:50), β-tubulin and anti-VGSCs (1:600), Kv1.1 (1:200), Kv1.2 (1:400), and -casp3/paranodin (rabbit, 1:1000; mouse, 1:2000) antibodies, followed by Alexa-conjugated secondary antibodies. Images were collected with a confocal microscope (model LSM 510; Carl Zeiss MicroImaging, Inc.) using Plan-Apochromat 63× or 100×/1.4 Oil DIC lenses. Image processing and morphometry were performed with the Metamorph software (Universal Imaging Corp.). Compared images were acquired in the same conditions. For cochlea imaging, mice were killed by cervical dislocation; the inner ear was removed and fixed by immersion in 1% PFA. Next, the cochlea was dissected out, cut into three coils, and incubated overnight at 4°C with antibodies.

EM

Anesthetized βIVΣ1 →−/− and +/+ mice were perfused transcardially with 2% PFA, 5% glutaraldehyde in 0.1 M cacodylate buffer, 1 mM CaCl₂, pH 7.4. Optic and sciatic nerves were prepared for EM as described previously (Traka et al., 2003). Contrasted ultrathin sections were observed with an electron microscope (model EM906; Carl Zeiss MicroImaging, Inc.). Morphometry was done on 15 micrographs of sciatic nerves for each genotype. The ratio between the width of NR and juxtaparanodes [/(node width/ juxtaparanode width) × 100] was calculated for each NR.
ABR
Anesthetized 8-wk-old βIVδ1 −/− mice and +/+ were exposed to tone bursts (4/6/8/12/16/24/32 kHz, 10 ms plateau with 1 ms cos2 onset and offset) or clicks of 0.03 ms generated by a System 2 (Tucker-Davis Technology) driving a high frequency speaker (Monacor). Intensities are shown as sound pressure level (dB root mean square for tone bursts, dB peak equivalent for clicks). The difference potential between vertex and mastoid was amplified (factor 5e4), filtered (low pass: 4 kHz, high pass: 100 Hz), and averaged (2 × 2,000 traces) to obtain two mean ABR traces for sound intensity. ABR latencies were analyzed after stimulation with 80-dB clicks, and the thresholds were estimated with a 10-dB precision by visual inspection. In some experiments, stimuli at 20 and 90 Hz and a recording time of 10 ms were used to compare the latency of wave IV on presentation of click stimuli at various intensities at slow and rapid stimulation rates.

Statistics
Statistics were performed with Sigma Stat 3.0 (SPSS Inc.). Data are reported as means ± SEM and compared using the unpaired Mann and Whitney U test for the immunostaining measurements and t test for the EM and ABR.

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