Specific Role of the Truncated βIV-Spectrin Σ6 in Sodium Channel Clustering at Axon Initial Segments and Nodes of Ranvier

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At axon initial segments and nodes of Ranvier in neurons, the spectrin membrane skeleton plays roles in physically stabilizing the plasma membrane integrity and in clustering voltage-gated sodium channels for proper conduction of the action potential. βIV-Spectrin, an essential component of the membrane skeleton at these sites, has an N-terminal-truncated isoform, Σ6, which is expressed at much higher levels than the full-length isoform Σ1. To investigate the role of βIV-spectrin Σ6, we generated Σ1-deficient mice with a normal level of Σ6 expression (Σ6+/− mice), and compared their phenotypes with those of previously generated mice lacking both Σ1 and Σ6 (Σ1Σ6−/− mice). The gross neurological defects observed in Σ1Σ6−/− mice, such as hindleg contraction, were apparently ameliorated in Σ1−/− mice. At cellular levels, Σ1Σ6−/− and Σ1−/− neurons similarly exhibited waving and swelling of the plasma membrane at axon initial segments and nodes of Ranvier. By contrast, the levels of ankyrin G and voltage-gated sodium channels at these sites, which are significantly reduced in Σ1Σ6−/− mice, were substantially recovered in Σ1−/− mice. We conclude that the truncated βIV-spectrin isoform Σ6 plays a specific role in clustering voltage-gated sodium channels, whereas it is dispensable for membrane stabilization at axon initial segments and nodes of Ranvier.

The spectrin membrane skeleton is a polygonal cytoskeletal meshwork attached to the cytoplasmic face of the plasma membrane (1). The basic unit of the spectrin skeleton is a heterotetramer of two α-spectrin and two β-spectrin proteins. β-Spectrin has an N-terminal actin-binding domain, and the spectrin tetramers are bound to one another indirectly via short actin filaments to form the meshwork. β-Spectrin also binds to a membrane adaptor protein ankyrin via the spectrin repeat 15 (2), thereby allowing the attachment of the spectrin-actin meshwork to the plasma membrane. Two major roles are known for the spectrin skeleton. One is to physically stabilize the plasma membrane integrity. In hereditary diseases with mutations in the erythrocyte-specific αL- and βL-spectrin genes, the erythrocyte membrane becomes fragile, resulting in elliptocytosis and spherocytosis, and eventually hemolytic anemia (3). The second role is to cluster specific integral membrane proteins at high density in specialized regions of the plasma membrane. For example, αL- and βL-spectrins stabilize the clustering of Na+/K+-ATPase at cell-cell contact sites in polarized epithelial cells through ankyrin B-mediated interaction with Na+/K+-ATPase (4).

Axon initial segments (AIS) and nodes of Ranvier (NR) are specialized axonal domains of the neuron that play essential roles in the firing and amplification of the action potential. The AIS is a short axonal segment located adjacent to the cell body. Voltage-gated sodium channels (VGSC) are highly concentrated at this site and open in response to neurotransmitter-triggered excitatory post-synaptic potential, leading to the firing of the action potential (5, 6). Axons in vertebrate neurons are often wrapped with myelin sheaths produced by oligodendrocytes in the central nervous system and by Schwann cells in the peripheral nervous system to increase the conduction velocity of the action potential. The NR is a short naked part of the axon between the myelin sheaths (7, 8). VGSC are also clustered at NR and open in response to the action potential that has been conducted through the myelinated part of the axonal membrane. In myelinated axons, therefore, the action potential is amplified each time it passes NR so that it does not attenuate before arriving at the axon terminal.

βIV-Spectrin, one of the five β-spectrin family members in mammals, is a component of the spectrin skeleton at AIS and NR (9). At these sites, βIV-spectrin binds ankyrin G, an AIS- and NR-specific isoform of ankyrin (10, 11). Ankyrin G in turn binds to VGSC (12). Analyses of mutant mice lacking βIV-spectrin have revealed several roles of the spectrin skeleton at AIS and NR. First, the levels of ankyrin G and VGSC are reduced at AIS and NR in βIV-spectrin-deficient mice, indicating that through binding to ankyrin G, βIV-spectrin stabilizes the clus-

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2 The abbreviations used are: AIS, axon initial segment; NR, node of Ranvier; VGSC, voltage-gated sodium channel.
tering of VGSC at these sites (11). Second, the plasma membrane at NR is swollen, resulting in abnormal length and width of NR in the absence of Blv-spectrin (13). Therefore, the spectrin skeleton physically stabilizes the plasma membrane at NR. Finally, cytoplasmic vesicles are often accumulated around the nodal region in Blv-spectrin-deficient mice (13). Although the nature of these vesicles is unknown, this phenotype suggests a role for Blv-spectrin also in vesicular transport through the axon. Consequently, loss of the β-liv-spectrin function causes various neurological defects such as deafness, tremor, and muscle contraction in mice (11, 13, 14).

The classical β-spectrins consist of the N-terminal actin-binding domain, 17 tandem spectrin repeats, a variable region of spectrin repeats, is expressed at much higher levels than the isoform shown to be expressed as proteins, an N-terminal-truncated isoform lacking the actin-binding domain and the first 10 spectrin repeats, a variable region drawn into the cytoskeleton. Expression of the β1 isoform is regulated by the presence of the NR function. We demonstrate that Blv-spectrin suggests a unique membrane skeleton at AIS and NR. Although an essential feature for β1-spectrin among the five mammalian β-spectrins, because such a high level expression of truncated isoforms has not been found in other β-spectrins (1). Therefore, the presence of the β6 isoform in Blv-spectrin suggests a unique membrane skeleton at AIS and NR. Although an essential role for β1 in stabilizing the plasma membrane integrity at NR has been shown using mutant mice lacking β1 but not β6 (16), the role for β6 has not been investigated.

To study the role of the truncated Blv-spectrin isoform β6, we generated mice expressing β6 but not β1 (β1/−/− mice) and performed a side-by-side comparison of their phenotypes with those of previously generated mice lacking both β1 and β6 (β1β6/−/− mice; Ref. 11). If a defect in β1β6/−/− mice was to be rescued or ameliorated in β1/−/− mice, it could be attributed to the loss of β6 function. We demonstrate that β6 is specifically required for the clustering of VGSC at AIS and NR.

EXPERIMENTAL PROCEDURES

Derivation and Genotyping of Mutant Mice—The Blv-spectrin genomic locus was isolated from a 129/Sv genomic library using 5′ regions of cDNAs for β1 and β6. The β1-specific targeting vector was constructed using a neo expression cassette (PGKneo2DTA; Ref. 17). This targeting vector replaces a 4.6-kb genomic fragment containing exon 1 and part of exon 2, flanked by 1.6-kb PstI-PstI and 4.3-kb Apal-Apal genomic sequences derived from the cloned genomic DNA. The construct was electroporated into 129/S4-derived AK7 embryonic stem cells (17), and colonies were selected with G418. Homologous recombination events were screened by the PCR as described (17), using primers corresponding to the neo gene and a genomic sequence outside the targeting construct. Southern blotting was performed according to standard procedures as described (17). Tissue culture and blastocyst injections were performed as described (18). PCR genotyping of mice was performed on tail biopsy samples as described (17), using the following combination of primers: forward, 5′-AGGCTCTGAT-GTATGGGTGT-3′; reverse, 5′-ACTCCCACTGGCCACT-CAC-3′; and neo, 5′-GGATTGGGAAGACAATAGCAG-3′. β1β6/−/− mice were generated by gene trapping in embryonic stem cells as described (11). Both β1/−/− and β1β6/−/− mice were analyzed on the 129/S4 x C57BL/6 F1 genetic background.

Immunoprecipitation and Immunoblotting—Brains were homogenized in 1 ml of lysis buffer (20 mM Tris-HCl, pH 7.4, 140 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A). After removing insoluble materials by centrifugation at 12,000 × g for 15 min, the supernatants were incubated overnight with anti-A-spectrin antibody (5 μg; Santa Cruz Biotechnology, Santa Cruz, CA) coupled to protein A-Sepharose beads (GE Healthcare). The immunoprecipitates, as well as the total supernatants, were separated by SDS-polyacrylamide gel electrophoresis and transferred to the Trans-Blot polyvinylidene difluoride membrane (Bio-Rad). Transferred proteins were then detected by immunoblotting. Primary antibodies used were anti-Blv-spectrin (1:400; Ref. 11) and anti-A-spectrin (1:200; Santa Cruz Biotechnology) antibodies. Secondary antibodies were peroxidase-conjugated anti-chicken IgY (Jackson ImmunoResearch, West Grove, PA) and anti-rabbit IgG (GE Healthcare) antibodies. Blots were detected using the ECL reagent (GE Healthcare).

Immunofluorescence Staining—Brains and sciatic nerves were isolated from mice, fixed with 1% paraformaldehyde in phosphate-buffered saline for 30 min at room temperature, cryoprotected with 30% sucrose in phosphate-buffered saline, embedded in the Tissue-Tek OCT compound (Sakura Fine-technical, Tokyo, Japan), and sectioned at 10 μm using a cryostat. Sections were stained with chicken anti-Blv-spectrin (1:400; Ref. 11), mouse anti-ankyrin G (20 μg/ml; Santa Cruz Biotechnology), mouse anti-pan-sodium channel (10 μg/ml; Sigma), and rabbit anti-Na,1.6 (10 μg/ml; Alomone Labs, Jerusalem, Israel) antibodies. Secondary antibodies were Alexa 488- and Alexa 594-conjugated anti-chicken IgY, anti-mouse IgG, and anti-rabbit IgG antibodies (Molecular Probes, Eugene, OR). Fluorescence images were captured with a confocal microscope (Axiovert, Carl Zeiss, Oberkochen, Germany) using the LSM5 PASCAL system (Carl Zeiss).

Electron Microscopy—Mice were perfused with 2.5% glutaraldehyde and 4% paraformaldehyde in 100 mM phosphate buffer, pH 7.4. Optic nerves were removed, cut into small pieces, immersed in the same fixative for 1 h, and postfixed with 1% osmium tetroxide in phosphate buffer for 1 h. Fixed specimens were dehydrated in a graded series of ethanol and embedded in Quetol-812 (Nissin EM, Tokyo, Japan). Ultrathin sections were cut, counter-stained with uranyl acetate and lead citrate, and observed in an electron microscope (H-7500, Hitachi, Tokyo, Japan) at an accelerating voltage of 80 kV.

RESULTS

Organization of the Mouse Blv-Spectrin Gene—Schematic structures of the Blv-spectrin isoforms, β1 and β6, are depicted in Fig. 1A. Using mouse cDNA fragments corresponding to the N-terminal region of each of the two iso-
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![Image](image-url)

**FIGURE 1.** \( \Sigma 1 \)-specific disruption of \( \beta IV \)-spectrin in mice. A, schematic structures of the \( \beta IV \)-spectrin isoforms. \( \Sigma 1 \) and \( \Sigma 6 \). ABD, actin-binding domain; PH, pleckstrin homology domain; VR, variable region. B, organization of the mouse \( \beta IV \)-spectrin gene. Exons are boxed. Open, shaded, and closed boxes represent the 5' non-coding region for \( \Sigma 1 \) mRNA, three distinct 5' non-coding regions for \( \Sigma 6 \) mRNAs, and protein-coding regions, respectively. The translation initiation codons (ATG) for \( \Sigma 1 \) and \( \Sigma 6 \) are located in exons 2 and 19, respectively. C, restriction map of the \( \Sigma 1 \)-specific gene targeting vector, as well as of the wild-type and mutant alleles. Arrowheads (F, R, and neo) indicate the positions of PCR primers used to genotype mutant mice as shown in E. DTA, diphtheria toxin A gene; neo\(^5\), neo-resistance gene; A, Apal; H, HincII; P, PstI; D, genomic DNAs from wild-type (+/+) and \( \Sigma 1 \) heterozygous mutant (+/-) embryonic stem cells were digested with HincII and examined by Southern blotting using the 1.6-kb PstI-PstI fragment upstream of exon 1 as a probe. The wild-type and mutant alleles provide 10.5- and 1.8-kb fragments, respectively. E, PCR genotyping using tail DNAs from wild-type (+/+), \( \Sigma 1 \) heterozygous (+/-), and \( \Sigma 1 \) homozygous (-/-) mutant mice as templates. This PCR amplifies 581- and 523-bp diagnostic fragments for wild-type and mutant alleles, respectively.

forms, we isolated genomic DNA fragments encompassing the exons encoding the 5' region of the mRNAs for \( \Sigma 1 \) and \( \Sigma 6 \). The organization of these exons was determined by sequence analysis. Exon 1 of the \( \beta IV \)-spectrin gene corresponded to the 5' non-coding region of \( \Sigma 1 \) mRNA, and the translation initiation codon for \( \Sigma 1 \) was located in exon 2 (Fig. 1B). The ATG codon encoding methionine 1321 of \( \Sigma 1 \) in exon 19 is most likely to be the translation initiation codon for \( \Sigma 6 \) because it is the most upstream ATG in \( \Sigma 6 \) cDNAs (11). We previously identified three \( \Sigma 6 \) cDNAs with different 5' non-coding regions (11). Sequences corresponding to these non-coding regions were all found between exons 18 and 19 (Fig. 1B, 18b, 18c, and 18d), suggesting that the three \( \Sigma 6 \) mRNAs are transcribed from different promoters located between exons 18 and 19.

**Generation of \( \Sigma 1^{-/-} \) Mice**—To disrupt the expression of \( \Sigma 1 \) without affecting that of \( \Sigma 6 \), we constructed a gene targeting vector that replaces a 4.6-kb genomic sequence encompassing exon 1 and part of exon 2 (including the translation initiation codon for \( \Sigma 1 \)) with a neo cassette (Fig. 1C). Following electroporation of the construct into 129/Sv- or strain mouse embryonic stem cells and selection with G418, nine of ~100 colonies scored positive for homologous recombination by a PCR screening. Homologous recombination in these clones was confirmed by genomic Southern blotting (Fig. 1D). Germ-line chimeras were derived from one of the clones and crossed to C57BL/6J mice to derive mutants. Heterozygous offspring did not display an overt phenotype.

Heterozygous mutant mice were intercrossed to derive \( \Sigma 1^{-/-} \) mice. Genotyping of the offspring by PCR using tail DNAs as templates (Fig. 1E) showed that the homozygous mice were recovered according to Mendelian expectations. To examine the expression of \( \Sigma 1 \) and \( \Sigma 6 \) in the homozygous mutants, brain lysates from wild-type and \( \Sigma 1^{-/-} \) mice, as well as from a \( \Sigma 1\Sigma 6^{-/-} \) mouse (11), were immunoblotted with anti-\( \beta IV \)-spectrin antibody. This antibody recognizes the variable region of \( \beta IV \)-spectrin, which is present in both \( \Sigma 1 \) and \( \Sigma 6 \) (Fig. 1A). The expression of \( \Sigma 1 \) was completely abolished in the \( \Sigma 1^{-/-} \) brain (Fig. 2A, top). By contrast, the level of \( \Sigma 6 \) expression in \( \Sigma 1^{-/-} \) brain was comparable with that in the wild type (Fig. 2A, top), indicating that \( \Sigma 6 \) is not destabilized in the absence of the \( \Sigma 1 \)-based classical spectrin meshwork.

**Both \( \Sigma 1 \) and \( \Sigma 6 \) Form a Complex with \( \alpha II \)-Spectrin in the Brain**—\( \alpha II \)-Spectrin is most likely the heterotetramerization partner for \( \beta IV \)-spectrin at the membrane skeleton of AIS and NR, because \( \alpha II^{-/-} \), but not \( \alpha I^{-/-} \), spectrin is expressed in neurons (19). We examined the level of \( \alpha II^{-/-} \) spectrin in both \( \Sigma 1^{-/-} \) and \( \Sigma 1\Sigma 6^{-/-} \) brains. Immunoblotting of the brain lysates with anti-\( \alpha II \)-spectrin antibody showed that \( \alpha II^{-/-} \) spectrin is expressed at comparable levels in wild-type, \( \Sigma 1^{-/-} \), and \( \Sigma 1\Sigma 6^{-/-} \) brains (Fig. 2A, bottom). To examine whether \( \Sigma 1 \) and \( \Sigma 6 \) interact with \( \alpha II \)-spectrin in neurons, we next performed

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5 Y. Uemoto, S.-i. Suzuki, and M. Komada, unpublished data.
results suggested that full-length isoform spectrin meshwork with precipitated from wild-type lysate (Fig. 2). They were not consistently detected in the total lysate and anti-spectrin antibodies. Bands indicated by asterisks are nonspecific, as they were not consistently detected in the total lysate and anti-spectrin immunoprecipitate.

**FIGURE 2. Interaction of Σ1 and Σ6 with all-spectrin.** A, immunoblot analysis of brain lysates from wild-type, Σ1−/−, and Σ1Σ6−/− mice with anti-βIV-spectrin (top) and anti-all-spectrin (bottom) antibodies. The positions of Σ1 and Σ6 are indicated. B, all-spectrin was immunoprecipitated from brain lysates of wild-type, Σ1−/−, and Σ1Σ6−/− mice, and the immunoprecipitates were immunoblotted with anti-βIV-spectrin (top) and anti-all-spectrin (bottom) antibodies. Bands indicated by asterisks are nonspecific, as they were not consistently detected in the total lysate and anti-spectrin immunoprecipitate.

**FIGURE 3. Σ6 localization and membrane instability at Σ1−/− AIS and NR.** Cryosections of the cerebral cortex (A and B), hippocampus (C and D), and sciatic nerve (E and F) from wild-type (A, C, and E) and Σ1−/− (B, D, and F) mice were stained with anti-βIV-spectrin antibody. Arrowheads in B and D indicate uneven staining by the antibody. Mice were sacrificed at 3 months of age. Bars, 10 μm (A–D) and 2 μm (E and F).

Neurological Disorders in Σ1−/− Mice—Throughout this study, wild-type, Σ1−/−, and Σ1Σ6−/− mice were all examined on the same 129/S4 x C57BL/6 F1 hybrid genetic background. Like Σ1Σ6−/− mice (11), Σ1−/− mice exhibited tremor that was first observable at weaning. However, it was apparently milder than that of Σ1Σ6−/− mice. As they become older (usually >8 months old), Σ1Σ6−/− mice often exhibit continuous contraction of the hindleg muscle (Ref. 11; supplemental Fig. S1). This phenotype was never observed in more than 50 Σ1−/− mice, which were older than 8 months (supplemental Fig. S1). In addition, Σ1Σ6−/− male mice are sterile (11). This is most likely due to a neurological defect that affects sexual behavior, because the histology of their reproductive tissues (testis and epididymis) as well as the morphology and movement of isolated sperms appeared normal, and the mutant mice showed no sign of copulation with wild-type females as judged by the presence of vaginal plugs. When four Σ1−/− male mice were crossed with wild-type females, by contrast, they all exhibited normal fertility. Overall, the gross phenotypes observed in Σ1Σ6−/− mice were rescued or milder in Σ1−/− mice, indicating that Σ6 is essential for normal neuronal functions.

**Σ1-independent Localization of Σ6 to AIS and NR**—Using the antibody against the variable region of βIV-spectrin (Fig. 1A), we examined the localization of Σ6 in Σ1−/− mice by immunofluorescence staining of tissue sections. Σ6 localized to AIS in the cerebral cortex and hippocampus (Fig. 3, B and D), as well as in other regions, of Σ1−/− brain. As expected from the much higher expression level of Σ6 than Σ1 in the brain (Ref. 11; Fig. 2A), the level of anti-βIV-spectrin staining was not significantly reduced in Σ1−/− mice compared with that in wild-type mice (Fig. 3, A–D). Also in Σ1−/− sciatic nerve axons, Σ6 localized normally to NR, although the level of anti-βIV-spectrin staining was lower than in wild-type neurons (Fig. 3, E and F). These results suggested that the localization of Σ6 to AIS and NR is regulated by a Σ1-independent mechanism.

**Membrane Destabilization at AIS in Σ1−/− Mice**—Although Σ6 localized to AIS, the anti-βIV-spectrin staining pattern of Σ6 was different between wild-type and Σ1−/− neurons both in the cerebral cortex and in the hippocampus. Whereas wild-type neurons were smoothly and uniformly stained, Σ1−/− neurons exhibited waving AIS (Fig. 3, B and D). In addition, staining levels were uneven in different regions of individual AIS (Fig. 3, B and D, arrowheads). The same wavy phenotype was observed when Σ1−/− AIS in the cerebral cortex were stained with antibodies against ankyrin G and VGSC (Fig. 5, B′
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and $E'$). These results suggested that the AIS membrane, which is defined by the presence of Σ6, is destabilized in the absence of $\Sigma 1$, and therefore that the full-length isoform $\Sigma 1$ is essential for maintenance of membrane stability at AIS.

Membrane Destabilization and Vesicle Accumulation at NR in $\Sigma 1^{−/−}$ Mice—The membrane architecture of NR in the central and peripheral nervous systems has been studied using electron microscopy in βIV-spectrin-deficient mice (13, 16). We compared the ultrastructure of NR in optic nerves of our $\Sigma 1\Sigma 6^{−/−}$ and $\Sigma 1^{−/−}$ mice in similar experiments. As reported, the NR plasma membrane of $\Sigma 1\Sigma 6^{−/−}$ and $\Sigma 1^{−/−}$ mice exhibited a similar wavy and swollen phenotype (Fig. 4, B and C, compare with the normal membrane in Fig. 4A). Moreover, the fuzzy electron-dense undercoating of the nodal membrane in wild-type mice (Fig. 4A', arrow) was absent in $\Sigma 1\Sigma 6^{−/−}$ as well as $\Sigma 1^{−/−}$ mice (Fig. 4, B' and C', arrows). These similar defects in $\Sigma 1\Sigma 6^{−/−}$ and $\Sigma 1^{−/−}$ NR suggested that also at NR, $\Sigma 1$ but not Σ6 plays an essential role in stabilizing the membrane integrity.

The other nodal phenotype in βIV-spectrin-deficient mice is the accumulation of cytoplasmic vesicular structures around the swollen NR membrane (13, 16). However, whether the loss of Σ6 is involved in this phenotype is unclear because the frequency of the appearance of such vesicles has not been compared between mice lacking both Σ1 and Σ6 and those lacking only Σ1. We therefore quantified the proportion of vesicle-associated NR (Fig. 4D, left), as well as the number of vesicles found around a single NR (Fig. 4D, right), in wild-type, $\Sigma 1^{−/−}$, and $\Sigma 1\Sigma 6^{−/−}$ optic nerves. For each genotype, 45 NR from three mice (15 NR from each) were examined. These parameters were not significantly different between wild-type and $\Sigma 1^{−/−}$ NR, but were much higher in $\Sigma 1\Sigma 6^{−/−}$ NR. These results suggested that the formation of cytoplasmic nodal vesicles in $\Sigma 1\Sigma 6^{−/−}$ optic nerves is due largely, if not totally, to the lack of Σ6.

Localization of Ankyrin G and VGSC at AIS in $\Sigma 1^{−/−}$ Mice—We next compared the levels of ankyrin G and VGSC at AIS in wild-type, $\Sigma 1^{−/−}$, and $\Sigma 1\Sigma 6^{−/−}$ mice by staining brain sections with anti-ankyrin G and anti-pan-sodium channel antibodies.

At AIS of the cerebral cortex in $\Sigma 1^{−/−}$ mice, levels of both ankyrin G (Fig. 5B') and VGSC (Fig. 5E') were slightly lower than in wild-type mice (Fig. 5, A' and D'). However, they were significantly higher than those in $\Sigma 1\Sigma 6^{−/−}$ mice (Fig. 5, C' and F'). In addition, we found that the ankyrin G- and VGSC-positive regions of axons are apparently longer in $\Sigma 1\Sigma 6^{−/−}$ neurons than in wild-type neurons (Fig. 5, C' and F', compared with A' and D'). No such abnormality was observed in $\Sigma 1^{−/−}$ neurons (Fig. 5, B' and E'). Measurement of the lengths of ankyrin G-positive regions on microscopic images showed that they are 25 ± 4, 23 ± 3, and 45 ± 4 μm (mean ± S.D.) for wild-type ($n = 24$ from three mice), $\Sigma 1^{−/−}$ ($n = 11$ from two mice), and $\Sigma 1\Sigma 6^{−/−}$ ($n = 22$ from three mice) neurons, respectively (Fig. 5G).

Also at AIS of Purkinje and granular neurons in the cerebellum, the levels of ankyrin G and Na$_{1.6}$ in $\Sigma 1^{−/−}$ mice (supplemental Fig. S2, B, B', E, and E') were lower than in wild-type (supplemental Fig. S2, A, A', D, and D') but higher than in $\Sigma 1\Sigma 6^{−/−}$ mice (supplemental Fig. S2, C, C', F, and F'). Na$_{1.6}$ is the major isoform of the VGSC β-subunit at AIS and NR (11, 20–22). Here we detected Na$_{1.6}$ because the anti-pan-sodium channel antibody did not efficiently stain AIS of cerebellar neurons. The elongation of ankyrin G- and VGSC-positive axonal regions, however, was not observed in Purkinje neurons in $\Sigma 1\Sigma 6^{−/−}$ mice (supplemental Fig. S2).

Localization of VGSC at NR in $\Sigma 1^{−/−}$ Mice—We next examined the localization of Na$_{1.6}$ at NR in sciatic nerve axons. The anti-pan-sodium channel antibody also failed to stain the NR at detectable levels. The number of Na$_{1.6}$-positive NR was reduced in $\Sigma 1^{−/−}$ mice (Fig. 6B') compared with wild-type mice (Fig. 6A'). However, it was higher than that in $\Sigma 1\Sigma 6^{−/−}$ mice (Fig. 6C'). To quantify the reduction of the Na$_{1.6}$-positive NR in the mutants, the number of Na$_{1.6}$-positive NR in the sciatic nerve sections was counted under the microscope. Counting in nine randomly chosen fields (0.2 mm$^2$ each) from three mice of each genotype revealed that in $\Sigma 1^{−/−}$ and $\Sigma 1\Sigma 6^{−/−}$ axons, the number is reduced to 62 ± 17 and 34 ± 8% (mean ± S.D.), respectively, of that in wild-type axons (Fig. 6D).

We were not able to examine the level of ankyrin G at NR because all of the tested anti-ankyrin G antibodies exhibited high nonspecific staining of the myelin sheath.3
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DISCUSSION

Whereas analyses of βIV-spectrin Σ1-specific knock-out mice have revealed an essential role for the full-length isoform in stabilizing the membrane integrity at NR (16), the role and significance of the truncated isoform Σ6 have been totally unclear. Through a side-by-side comparison of Σ1/Σ6−/− and Σ1−/− mice on the same genetic background, we here demonstrate that Σ6 plays a specific role in the clustering of VGSC at AIS and NR.

Σ6 at AIS of Σ1−/− cerebral and hippocampal neurons exhibited a wavy and uneven staining pattern with anti-βIV-spectrin (Fig. 3). The same pattern was observed when AIS were stained for other AIS-specific membrane proteins, ankyrin G and VGSC (Fig. 5). These results suggest that in the absence of Σ1, the AIS membrane is ruffled and the cytoplasmic face of the membrane is unequally attached with the putative membrane skeleton composed of βIV-spectrin Σ6 and its binding partner αII-spectrin (2). Unlike in the cerebral and hippocampal neurons, the abnormal staining pattern of AIS with anti-βIV-spectrin, anti-ankyrin G, and anti-VGSC antibodies was not apparent in cerebellar Purkinje neurons in Σ1−/− mice (supplemental Fig. S2). The reason for this difference is unclear. One possibility might be that sensitivity of membrane stability to the loss of βIV-spectrin Σ1 varies among the AIS of different neurons.

As reported for other βIV-spectrin-deficient mice (13, 16), the electron-dense fuzzy cytoplasmic undercoating was missing from the NR membrane of optic nerve axons also in our Σ1/Σ6−/− and Σ1−/− mice (Fig. 4). This was accompanied by swelling of the NR membrane in these axons (Fig. 4). Taken together, the abnormal membrane architecture at AIS and NR of Σ1/Σ6−/− and Σ1−/− mice suggests that Σ6 is dispensable for the stabilization of membrane integrity and that the classical spectrin meshwork formed by the full-length βIV-spectrin Σ1 and αII-spectrin plays an essential role. This is consistent with the situation in erythrocytes. The erythrocyte-specific βI-spectrin, which does not have an abundantly expressed truncated isoform like βIV-spectrin Σ6 (23), is capable of stabilizing the membrane integrity in these cells.

In optic nerve of Σ1/Σ6−/− mice, vesicular structures were often accumulated in the axonal cytoplasm in close proximity to NR (Fig. 4). The frequency of the appearance of such vesicles in Σ1−/− mice was much lower than that in Σ1/Σ6−/− mice and was similar to that in wild-type mice (Fig. 4). These results indicate that the phenotype is largely rescued by the expression of Σ6, thus suggesting a Σ6-specific function. A previous study showed that also in Σ1−/− mice, vesicles are often accumulated around NR in sciatic nerves (16). However, such accumulation of nodal vesicles was not demonstrated in optic nerves (16). Therefore, the degree of the phenotype might differ between the central and peripheral nervous systems. The identity of the vesicles is currently unknown. One possibility is that they are transport vesicles that deliver specific membrane proteins to NR and that their access to or fusion with the NR membrane is perturbed in the absence of Σ6. It is important in future studies to elucidate...
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βIV-spectrin Na⁺,1.6

wild-type Σ1

One explanation for this phenotype is that in the absence of Σ6, the destabilized membrane protein cluster diffuses toward the axon terminal from AIS. These results therefore further support the role of Σ6 in maintaining the clustering of VGSC at AIS and NR. A similar phenotype has been reported for Purkinje neurons in cerebellum-specific ankyrin G-deficient mice. In these mice, the AIS region positive for neurofascin 186, an AIS-specific cell adhesion molecule, is elongated at variable levels (24). In our βIV-spectrin Σ6 mice, however, this phenotype was not apparent in Purkinje neurons (supplemental Fig. S2). The reason for this discrepancy is unclear. How intensely this membrane skeletal function depends on βIV-spectrin Σ6 and ankyrin G may vary among neural cell types.

The gross neurological defects observed in Σ1Σ6 mice, such as the tremor, hindleg contraction, and impaired sexual behavior in male, were ameliorated or not observed in Σ1 mice (see “Results” for details). This was correlated with the levels of ankyrin G and VGSC at AIS and NR, which were higher in Σ1 mice than in Σ1Σ6 mice (Figs. 5, 6, and supplemental Fig. S2). On the other hand, it was not correlated with the level of membrane destabilization at AIS and NR, which was similarly observed in Σ1 mice and Σ6 mice (Figs. 3–5). Therefore, the neurological disorders in Σ1Σ6 mice are likely to be due to the reduced level of VGSC at AIS and NR that probably results in insufficient firing and amplification of the action potential, rather than to the membrane fragility at these specific neuronal domains.

Co-immunoprecipitation experiments showed that Σ6 interacts with αII-spectrin (Fig. 2). In the spectrin heterotetramer, α- and β-spectrins bind to each other in two ways. The anti-parallel lateral association is mediated by the binding of the first two spectrin repeats of β-spectrin with the last two spectrin repeats of α-spectrin (25, 26). The head to head association is mediated by the interaction between the last incomplete spectrin repeat of β-spectrin with the N-terminal region of α-spectrin (27, 28). As Σ6 harbors the last spectrin repeat but not the first two, it may form a head to head heterodimer with αII-spectrin at AIS and NR. However, how Σ6 fits in the membrane skeleton with αII-spectrin at AIS and NR is unclear. It is also unknown why such a truncated isoform, besides the full-length S1, is required for the high density clustering of VGSC. These questions remain to be addressed in future studies. Nonetheless, the results presented here demonstrate an essential role for the unique truncated isoform of βIV-spectrin in neuronal function.

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