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# Developmental Changes in Expression of $\beta$ IV Spectrin Splice Variants at Axon Initial Segments and Nodes of Ranvier

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Axon initial segments (AIS) and nodes of Ranvier are highly specialized axonal membrane domains enriched in Na<sup>+</sup> channels. These Na<sup>+</sup> channel clusters play essential roles in action potential initiation and propagation. AIS and nodal Na<sup>+</sup> channel complexes are linked to the actin cytoskeleton through  $\beta$ IV spectrin. However, neuronal  $\beta$ IV spectrin exists as two main splice variants: a longer  $\beta$ IV $\Sigma$ 1 variant with canonical N-terminal actin and  $\alpha$ II spectrin-binding domains, and a shorter  $\beta$ IV $\Sigma$ 6 variant lacking these domains. Here, we show that the predominant neuronal  $\beta$ IV spectrin splice variant detected in the developing brain switches from  $\beta$ IV $\Sigma$ 1 to  $\beta$ IV $\Sigma$ 6, and that this switch is correlated with expression changes in ankyrinG (ankG) splice variants. We show that  $\beta$ IV $\Sigma$ 1 is the predominant splice variant at nascent and developing AIS and nodes of Ranvier, but with increasing age and in adults  $\beta$ IV $\Sigma$ 6 becomes the main splice variant. Remarkably, super-resolution microscopy revealed that the spacing of spectrin tetramers between actin rings remains unchanged, but that shorter spectrin tetramers may also be present. Thus, during development  $\beta$ IV spectrin may undergo a switch in the splice variants found at AIS and nodes of Ranvier.

**Keywords:** cytoskeleton, spectrin, axon, node of Ranvier, axon initial segment, ankyrin

## INTRODUCTION

Neurons are highly polarized cells comprised of two structurally and functionally distinct domains: the axon and dendrites (Craig and Banker, 1994). The polarization of neurons allows for the unidirectional flow of information from dendrites/soma to axons. The dendrites and soma receive the upstream synaptic inputs; these are integrated and the decision to fire an action potential is made at the axon initial segment (AIS; Yoshimura and Rasband, 2014). AIS are characterized by high densities of voltage-gated Na<sup>+</sup> and K<sup>+</sup> channels that function to initiate and modulate action potentials (Kole and Stuart, 2012). In myelinated axons of vertebrates, action potentials propagate through the opening of Na<sup>+</sup> channels at nodes of Ranvier.

The AIS and nodes of Ranvier have a common molecular organization; in addition to the clustering of Na<sup>+</sup> and K<sup>+</sup> channels, these two regions share an enrichment of adhesion molecules and molecular scaffolds (Chang and Rasband, 2013). AnkyrinG (ankG) and  $\beta$ IV spectrin are the main components of the cytoskeleton at the AIS and nodes of Ranvier in neurons

(Kordeli et al., 1995; Berghs et al., 2000). AnkG interacts with and clusters membrane proteins (e.g., Na<sup>+</sup> channels) and  $\beta$ IV spectrin, while  $\beta$ IV spectrin is thought to link the ankG/Na<sup>+</sup> channel membrane protein complex to the actin cytoskeleton (Yang et al., 2007; Ho et al., 2014). Loss of ankG disrupts AIS assembly and neuronal function (Zhou et al., 1998). In  $\beta$ IV spectrin deficient mice, ankG and Na<sup>+</sup> channel densities are reduced at the AIS, probably due to diminished stability of the complex (Komada and Soriano, 2002). Thus, both ankG and  $\beta$ IV spectrin are essential for proper AIS ion channel complexes and axon domain organization.

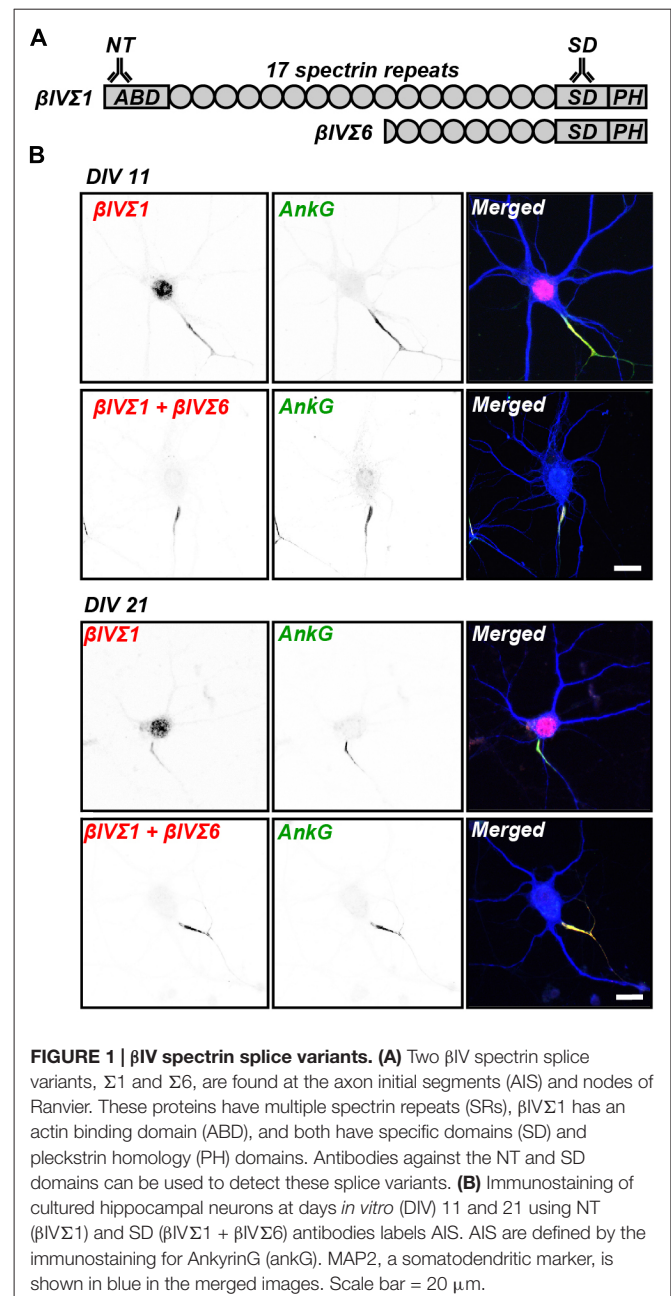
Alternative splicing generates six  $\beta$ IV spectrin splice variants ( $\beta$ IV $\Sigma$ 1– $\beta$ IV $\Sigma$ 6; Berghs et al., 2000; Komada and Soriano, 2002). Two variants,  $\beta$ IV $\Sigma$ 1 and  $\beta$ IV $\Sigma$ 6, are thought to be at the AIS and nodes of Ranvier (Komada and Soriano, 2002; Lacas-Gervais et al., 2004).  $\beta$ IV $\Sigma$ 1 is the largest of the splice variants and consists of an N-terminal actin-binding domain, 17 spectrin repeats (SRs), a “specific” domain (SD) and a pleckstrin homology (PH) domain (Figure 1A).  $\beta$ IV $\Sigma$ 6 is an N-terminal truncation that lacks the actin-binding domain, SRs 1–9, and part of SR 10 (Figure 1A). Spectrins are thought to function as heterotetramers consisting of two  $\alpha$  and two  $\beta$  spectrin subunits, with the minimal lateral interactions occurring between the first two triple-helical domains of  $\beta$ -spectrins and the last two triple helical domains of  $\alpha$ -spectrins (Speicher et al., 1992). Thus, it is not clear how  $\beta$ IV $\Sigma$ 6 would function in a heterotetramer. Due to the poorly understood expression patterns of the  $\beta$ IV spectrin splice variants, their individual roles remain unclear. Although the  $\sim$ 190 nm spacing of  $\beta$ IV spectrin as revealed by super-resolution microscopy suggests they function as tetramers in the AIS, no  $\alpha$  spectrin has yet been reported at the AIS (Galiano et al., 2012; Xu et al., 2013; Leterrier et al., 2015).

Here, we examined the spatial and temporal expression patterns of  $\beta$ IV $\Sigma$ 1 and  $\beta$ IV $\Sigma$ 6 at the AIS and nodes of Ranvier. We used anti- $\beta$ IV spectrin antibodies against the N-terminal region that detects  $\beta$ IV $\Sigma$ 1, and antibodies against the SD that detects both  $\beta$ IV $\Sigma$ 1 and  $\beta$ IV $\Sigma$ 6 (Figure 1A). We found in early development that  $\beta$ IV $\Sigma$ 1 is the dominant splice variant at the AIS and nodes of Ranvier. However, as development proceeded, this shifted and  $\beta$ IV $\Sigma$ 6 became the dominant isoform in adult mice. Finally, stochastic optical reconstruction microscopy (STORM) super-resolution microscopy revealed that the pattern of  $\beta$ IV spectrin immunoreactivity remained periodic despite the switch in expression of these two splice variants, although a more complicated periodic pattern suggests the existence of shorter spectrin tetramers that may include  $\beta$ IV $\Sigma$ 6. Together, these studies reveal the differential expression and localization of  $\beta$ IV spectrin splice variants at the AIS and nodes of Ranvier during development of the nervous system.

## MATERIALS AND METHODS

### Animals

Sprague Dawley rats were obtained from Harlan Sprague Dawley (Indianapolis, IN, USA). C57BL/6 mice were from Charles River



**FIGURE 1 |  $\beta$ IV spectrin splice variants.** (A) Two  $\beta$ IV spectrin splice variants,  $\Sigma$ 1 and  $\Sigma$ 6, are found at the axon initial segments (AIS) and nodes of Ranvier. These proteins have multiple spectrin repeats (SRs),  $\beta$ IV $\Sigma$ 1 has an actin binding domain (ABD), and both have specific domains (SD) and pleckstrin homology (PH) domains. Antibodies against the NT and SD domains can be used to detect these splice variants. (B) Immunostaining of cultured hippocampal neurons at days *in vitro* (DIV) 11 and 21 using NT ( $\beta$ IV $\Sigma$ 1) and SD ( $\beta$ IV $\Sigma$ 1 +  $\beta$ IV $\Sigma$ 6) antibodies labels AIS. AIS are defined by the immunostaining for AnkyrinG (ankG). MAP2, a somatodendritic marker, is shown in blue in the merged images. Scale bar = 20  $\mu$ m.

Laboratories (Wilmington, MA, USA). Both male and female mice were used in these studies. All experiments were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee.

### Antibodies

Rabbit anti- $\beta$ IV spectrin NT, and rabbit or chicken anti- $\beta$ IV spectrin SD antibodies were previously described (Yang et al., 2004). The anti- $\beta$ IV spectrin NT antibody was generated against a synthetic peptide corresponding to amino acids 15–38 of the  $\beta$ IV $\Sigma$ 1 splice variant. Anti- $\beta$ IV spectrin SD

antibody was generated against the SD (Berghe et al., 2000). The goat anti-ankG antibody for immunoblot analysis was generated against the C-terminal domain of ankG and was kindly provided by Dr. Vann Bennett (Duke University, Durham, NC, USA; Ho et al., 2014). The following other primary antibodies were used: mouse monoclonal anti-ankG for immunostaining (N106/36, UC Davis/NIH NeuroMab facility, Davis, CA, USA); rabbit anti-GAPDH (Sigma-Aldrich, St. Louis, MO, USA); mouse monoclonal anti-Caspr (K65/35, UC Davis/NIH NeuroMab facility); and chicken polyclonal anti-MAP2 (EnCor Biotechnology, Gainesville, FL, USA) antibodies. Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA) and Life Technologies (Thermo Fisher Scientific, Waltham, MA, USA).

## Brain Lysate Preparation

Whole mouse brains were dissected and homogenized in ice-cold homogenization buffer (0.32M sucrose, 5 mM sodium phosphate, pH 7.4, 1 mM sodium fluoride and 1 mM sodium orthovanadate, containing 0.5 mM phenylmethylsulfonyl fluoride, 2  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml leupeptin, 2  $\mu$ g/ml antipain and 10  $\mu$ g/ml benzamide). Crude homogenates were then centrifuged at 600 $\times$  g for 10 min to remove debris and nuclei. The resulting supernatant was centrifuged at 30,000 $\times$  g for 90 min, then the pellet was re-suspended in ice-cold homogenization buffer. Protein concentrations were determined using the BCA method (Thermo Fisher Scientific, Waltham, MA, USA). After adding SDS sample buffer, the samples were subjected to SDS-PAGE and immunoblot analysis.

## RT-qPCR

RNA was isolated from whole mouse brains in triplicate using Trizol (Invitrogen, Carlsbad, CA, USA) and converted to cDNA with Superscript (Invitrogen) using a combination of oligo(T)s and random hexamers. mRNA was quantified with a Nanodrop spectrophotometer to ensure equal starting amounts. Gene specific primers that bridged consecutive exons were used to ensure detection of mRNA. For qPCR, using Power SYBR<sup>®</sup> Green PCR Master Mix (Applied Biosystems), the signal from every reaction at the end of each 60°C annealing extension step of each cycle was recorded on a CFX96 Real Time System (BioRad). Data are normalized to GAPDH. Primers used are as follows:  $\beta$ IV $\Sigma$ 1 (forward) TGAGGGCCCAGCAGTGGATGC;  $\beta$ IV $\Sigma$ 6 (forward) GACGCTCCTCCAACGCCG;  $\beta$ IV $\Sigma$ 1/ $\Sigma$ 6 (reverse) GGTGCCG GAGCCATCTCTTGT; 190 AnkG (forward) CTTTGCCCTCC TAGCTTTAC; 190 AnkG (reverse) TCTGTCCAATAAGTCC CAG; 270 AnkG (forward) GCCATGTCTCCAGATGTTG; (reverse) TCTGTCCAATAAGTCCCAG; 480 AnkG (forward) AGTAGGAGGACTGGTCCG; (reverse) AGTTGTGGCATTCT TTCCG.

## Culture of Hippocampal Neurons

Brains from embryonic day 18 rat embryos were collected into ice-cold HBSS without calcium or magnesium (Invitrogen,

Carlsbad, CA, USA). Embryonic hippocampi were dissected and collected in ice-cold HBSS. The collected tissue was incubated with 0.25% Trypsin in HBSS at 37°C for 15 min and washed with HBSS. After adding plating media (Neurobasal medium (Invitrogen) with 10% HyClone FetalClone III serum (Thermo Fisher Scientific, Waltham, MA, USA)), these hippocampi were mechanically dissociated using a fire-polished Pasteur pipette. The suspension was centrifuged for 5 min at 200 $\times$  g. The pelleted cells were briefly washed and resuspended in plating media. Neurons were plated on glass coverslips coated with poly-D-lysine (Sigma-Aldrich) at low density for immunocytochemistry (120 cells/mm<sup>2</sup>) and on plastic dishes with poly-D-lysine at high density for western blot (520 cells/mm<sup>2</sup>). After neurons were incubated in a humidified 5% CO<sub>2</sub> incubator at 37°C for 3 h, the media was exchanged to maintaining media (Neurobasal medium with 2% B-27 supplement (Invitrogen) and 2 mM GlutaMAX (Invitrogen)). The cultures were maintained by exchanging half of the volume of media twice a week with new maintaining media. For immunoblotting, neurons were washed with PBS, and collected with SDS sample buffer. The samples were subjected to SDS-PAGE and immunoblot analysis.

## Immunostaining and Imaging

Cultured neurons at 11 and 21 days *in vitro* (DIV) were fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.2) at 4°C for 30 min, followed by treatment with PBTGS (0.1M PB with 0.3% Triton X-100 (Sigma-Aldrich) and 10% goat serum (Invitrogen)) for 1 h at room temperature. For immunostaining of nervous system tissues, brains, optic and sciatic nerves were dissected at the indicated times, fixed in 4% PFA for 30 min for optic and sciatic nerves, or 1.5 h for brains; this was followed by immersion in 20% sucrose (w/v) in 0.1 M PB overnight at 4°C. The tissues were then frozen in Tissue-Tek OCT compound (Sakura Finetek, Tokyo, Japan) and sectioned using a Cryostat (CryoStar NX70, Thermo Fisher Scientific). Sections were collected and suspended in 0.1 M PB, then spread out on glass coverslips. The tissues were treated with PBTGS for 1 h at room temperature. The samples were incubated with primary antibodies diluted in PBTGS at room temperature overnight. Following this, samples were incubated with secondary antibodies for 1 h at room temperature. Immunofluorescence labeling was visualized, and images were collected on an AxioImager Z1 microscope (Carl Zeiss, Jena, Germany) fitted with an AxioCam digital camera (Carl Zeiss). AxioVision (Carl Zeiss) software was used for the collection and measurement of images. Images used for fluorescent signal quantification were collected using the same exposure settings across all animals and stages for the channels with  $\beta$ IV spectrin NT and  $\beta$ IV spectrin SD.

## STORM Imaging

After 13–28 days in culture, neurons were fixed using 4% PFA for 10 min. After blocking, they were incubated with primary antibodies overnight at 4°C, then with secondary antibodies for 1 h at room temperature. STORM imaging was performed on an



N-STORM microscope (Nikon Instruments, Melville, NY, USA). Coverslips were imaged in STORM buffer: Tris 50 mM (pH 8); NaCl 10 mM; 10% glucose; 100 mM MEA; 3.5 U/ml pyranose oxidase; and 40 mg/ml catalase. The sample was continuously illuminated at 647 nm (full power) and 30,000–60,000 images were acquired at 67 Hz, with progressive reactivation by simultaneous 405-nm illumination (Leterrier et al., 2015).

## Image Quantification

Images were analyzed using NIH ImageJ software. Mean fluorescent signal intensities of rabbit anti- $\beta$ IV spectrin NT and chicken anti- $\beta$ IV spectrin SD were quantified in raw images using a line scan along the length of the AIS or for a region of interest encompassing the area of the node of Ranvier. All visible AIS or nodes of Ranvier were measured in each image; these measurements were averaged for individual animals. Population means were calculated for all tissues and developmental stages. Measurements for the rabbit anti- $\beta$ IV spectrin NT antibody showed expression of  $\beta$ IV $\Sigma$ 1; the difference of the fluorescence intensities for the two  $\beta$ IV spectrin antibodies gave the relative expression of  $\beta$ IV $\Sigma$ 6. Three animals were used at each time point analyzed. All statistical comparisons were performed using Student's *t*-test. The number of AIS measured: P1 ( $n = 96$ ), P3 ( $n = 96$ ), P9 ( $n = 96$ ), 5-mo ( $n = 56$ ). Number of nodes of Ranvier measured in optic nerves: P9 ( $n = 115$ ), P15 ( $n = 80$ ), P30 ( $n = 80$ ), 5-mo ( $n = 80$ ). Number of nodes of Ranvier measured in sciatic nerves: P1 ( $n = 120$ ), P3 ( $n = 120$ ), P9 ( $n = 118$ ), 5-mo ( $n = 80$ ). Number of nodes of Ranvier measured in cerebellum: P9 ( $n = 141$ ), P15 ( $n = 90$ ), P30 ( $n = 102$ ), 5-mo ( $n = 114$ ).

## RESULTS

### $\beta$ IV $\Sigma$ 6 Is Highly Upregulated Following AIS Formation

Cultured hippocampal neurons are commonly used to study the development of neuronal polarity and assembly of unique domains (e.g., synapses, AIS, etc.; Galiano et al., 2012). To investigate the spatial expression patterns of  $\beta$ IV spectrin splice variants in neurons, we used two anti- $\beta$ IV spectrin antibodies generated against the NT and SD domains (Figure 1A; Yang et al., 2004). The anti- $\beta$ IV spectrin SD antibody recognizes both  $\Sigma$ 1 and  $\Sigma$ 6 variants, whereas the anti- $\beta$ IV spectrin NT antibody recognizes only the  $\Sigma$ 1 variant (Figure 1A). Immunostaining 11 and 21 DIV cultured hippocampal neurons with these antibodies showed both the SD and NT immunoreactivities colocalized with ankG at the AIS (Figure 1B). Since only the NT antibody showed any staining in the nucleus, but not the SD antibody that recognizes both splice variants, we conclude the nuclear NT immunoreactivity is nonspecific.

To determine the temporal expression profile of  $\beta$ IV spectrin splice variants, we performed immunoblotting using homogenates of cultured hippocampal neurons and developing brain (Figures 2A,B). Both in cultured neurons and in developing mouse brain the  $\beta$ IV $\Sigma$ 1 splice variant was the earliest splice variant observed. However, the expression levels

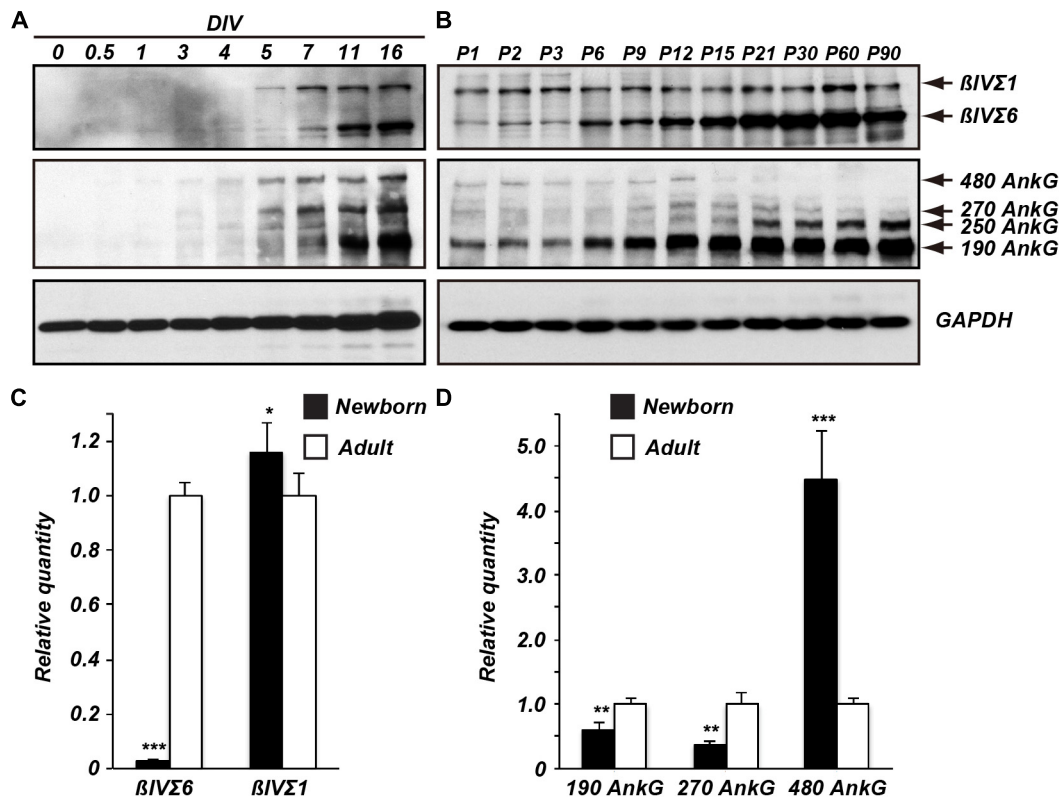
of  $\beta$ IV $\Sigma$ 6 dramatically increased at later time points both in culture and in the brain, indicating the most abundant form of  $\beta$ IV spectrin in mature neurons is the shorter  $\beta$ IV $\Sigma$ 6 splice variant.

$\beta$ IV spectrin is localized to the AIS through the binding of its 15th SR to ankG; unlike the actin-binding-domain, the ankG-binding domain is present in both  $\beta$ IV $\Sigma$ 1 and  $\beta$ IV $\Sigma$ 6 (Figure 1A; Yang et al., 2007). We examined the expression levels of ankG in cultured hippocampal neurons and in developing mouse brain (Figures 2A,B). The main ankG splice variants thought to be located at AIS and nodes of Ranvier are the 480 and 270 kDa isoforms (Kordeli et al., 1995). In cultured neurons all ankG splice variants increased with age (Figure 1A). In contrast, in the brain there was a dramatic increase in the ankG 190 kDa isoform, and an apparent reduction in 480 and 270 kDa splice variants. Although these same antibodies robustly label AIS in brain sections (data not shown), we speculate the apparent reduction in 480 and 270 kDa forms may reflect their extreme detergent insolubility and poor resolvability by SDS-PAGE.

To further define the differential expression of the  $\beta$ IV spectrin and ankG splice variants, we measured their transcript levels by reverse transcription-quantitative polymerase chain reaction (RT-qPCR; Figures 2C,D). Consistent with the immunoblotting results, the  $\beta$ IV $\Sigma$ 6 transcript levels from adult mice were significantly higher than those from newborn mice (Figure 2C). In contrast  $\beta$ IV $\Sigma$ 1 transcript levels from newborn mice were slightly higher when compared to adult mice. AnkG transcripts from adult mice showed that levels of ankG 190 and 270 kDa were increased relative to newborn mice, while the levels of ankG 480 transcripts were significantly lower than in newborn mice. These results indicate that transcript and protein expression levels of  $\beta$ IV spectrin and ankG splice variants reflect similar changes during mouse brain development. In particular,  $\beta$ IV $\Sigma$ 6 is dramatically increased during development, while 480 kDa ankG is reduced.

### $\beta$ IV Spectrin Splice Variants Change at the AIS and Nodes of Ranvier during Development

To determine if the change in  $\beta$ IV spectrin splice variant expression occurs specifically at AIS, we compared the expression levels of  $\beta$ IV $\Sigma$ 1 and  $\beta$ IV $\Sigma$ 6 at AIS in mouse cortex throughout development by double-immunolabeling with rabbit anti-NT ( $\beta$ IV $\Sigma$ 1) and chicken anti-SD ( $\beta$ IV $\Sigma$ 1 +  $\Sigma$ 6) antibodies. The SD immunoreactivity was localized at the AIS in both P1 and 5 month-old mouse cortex (Figure 3A). The NT immunoreactivity also labeled AIS and colocalized with the SD staining (Figure 3A; except for the non-specific nuclear immunoreactivity). Keeping all imaging parameters constant for each time point measured, we then subtracted the measured NT fluorescence intensity ( $\beta$ IV $\Sigma$ 1) from the measured SD fluorescence intensity ( $\beta$ IV $\Sigma$ 1 +  $\Sigma$ 6) which permitted us to estimate the relative contribution of  $\Sigma$ 6 to the SD immunostaining. We found that during early AIS assembly,  $\beta$ IV $\Sigma$ 1 is the predominant splice variant at AIS, but that this gradually changes such that by 5 months



**FIGURE 2 | Temporal expression of  $\beta$ IV spectrin and ankG splice variants.** Immunoblots of  $\beta$ IV spectrin and ankG from cultured neurons (A) and from brain homogenates (B). Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) of  $\beta$ IV spectrin (C) and ankG (D) splice variant transcripts detected in developing brain. Error bars  $\pm$  SEM. \* $p$  < 0.01, \*\* $p$  < 0.001, \*\*\* $p$  < 0.0001.

of age  $\beta$ IV $\Sigma$ 6 increases at the AIS relative to  $\beta$ IV $\Sigma$ 1 (Figure 3B).

Next, we performed a similar comparison of  $\beta$ IV $\Sigma$ 1 and  $\beta$ IV $\Sigma$ 6 immunostaining at nodes of Ranvier in sciatic nerve (Figures 4A,B), optic nerve (Figure 4C), and in cerebellum (Figure 4D). Remarkably, we found a similar shift at nodes from  $\beta$ IV $\Sigma$ 1 at new nodes of Ranvier to  $\beta$ IV $\Sigma$ 6 in older animals. These results suggest that while  $\beta$ IV $\Sigma$ 1 is the main  $\beta$ IV spectrin splice variant found at the AIS and nodes of Ranvier during early development, with increasing age  $\beta$ IV $\Sigma$ 1 may be replaced by the shorter  $\beta$ IV $\Sigma$ 6 splice variant. These observations are consistent with the results obtained by immunoblotting and RT-qPCR (Figure 2).

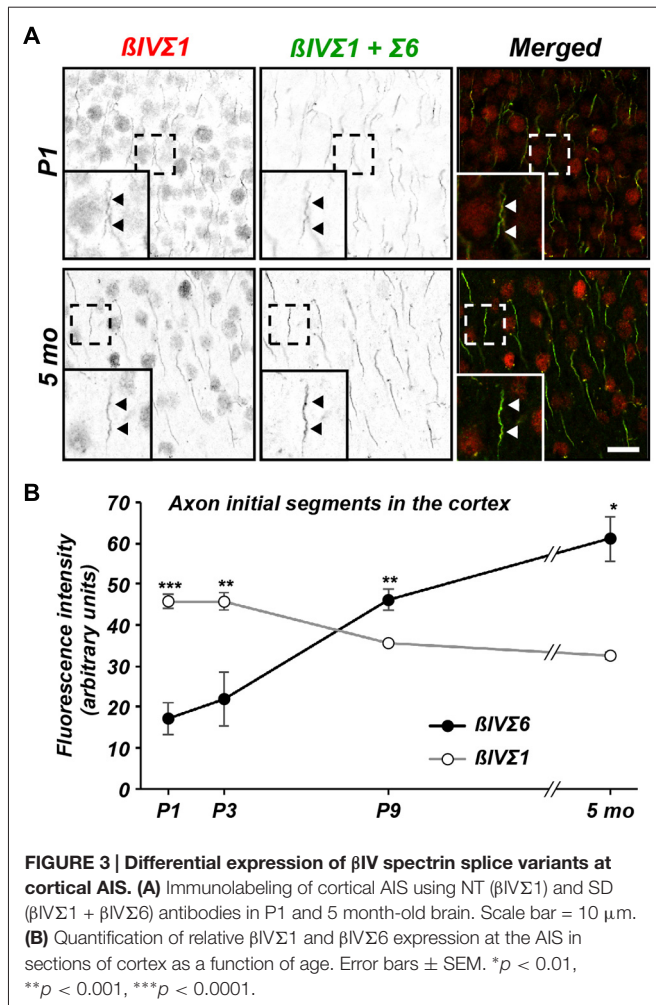
### Super-Resolution Imaging of $\beta$ IV Spectrin Distribution during Development

Recent experiments using STORM super-resolution microscopy revealed a periodic cytoskeleton at the AIS consisting of rings of actin spaced at  $\sim$ 190 nm intervals (Xu et al., 2013; Leterrier et al., 2015). Intriguingly, the spacing of these rings corresponds to the length of spectrin tetramers. Indeed, STORM imaging of  $\beta$ IV spectrin using our NT and SD antibodies revealed that the actin rings colocalized or alternate with the spectrin immunoreactivity,

respectively (Xu et al., 2013; Leterrier et al., 2015). Although the spacing of AIS actin rings has not yet been shown to depend on  $\beta$ IV spectrin, the spacing of actin rings in the distal axon does depend on  $\beta$ II spectrin (Zhong et al., 2014). Since the lengths of  $\beta$ IV $\Sigma$ 1 and  $\beta$ IV $\Sigma$ 6 are quite different, we wondered if the spacing of actin rings might change during development with the increased abundance of the shorter splice variant at AIS. To test this possibility, we performed STORM imaging using SD antibodies on cultured hippocampal neurons at DIV 13, 16, 20 and 28 (Figures 5A–D). Despite the increased protein and expression of the shorter  $\beta$ IV $\Sigma$ 6 splice variant, we still observed that the major peak of SD immunoreactivity occurred at an interval of  $\sim$ 190 nm in older neurons, suggesting that the spacing continues to be determined primarily by the  $\beta$ IV $\Sigma$ 1 splice variant. Nevertheless, secondary peaks of lesser intensity could be detected on intensity profiles that were  $55 \pm 2.67$  nm (SEM) to each side of the major peaks (see Figure 5C, asterisks). Importantly, these secondary peaks became more frequent and pronounced with increasing age.

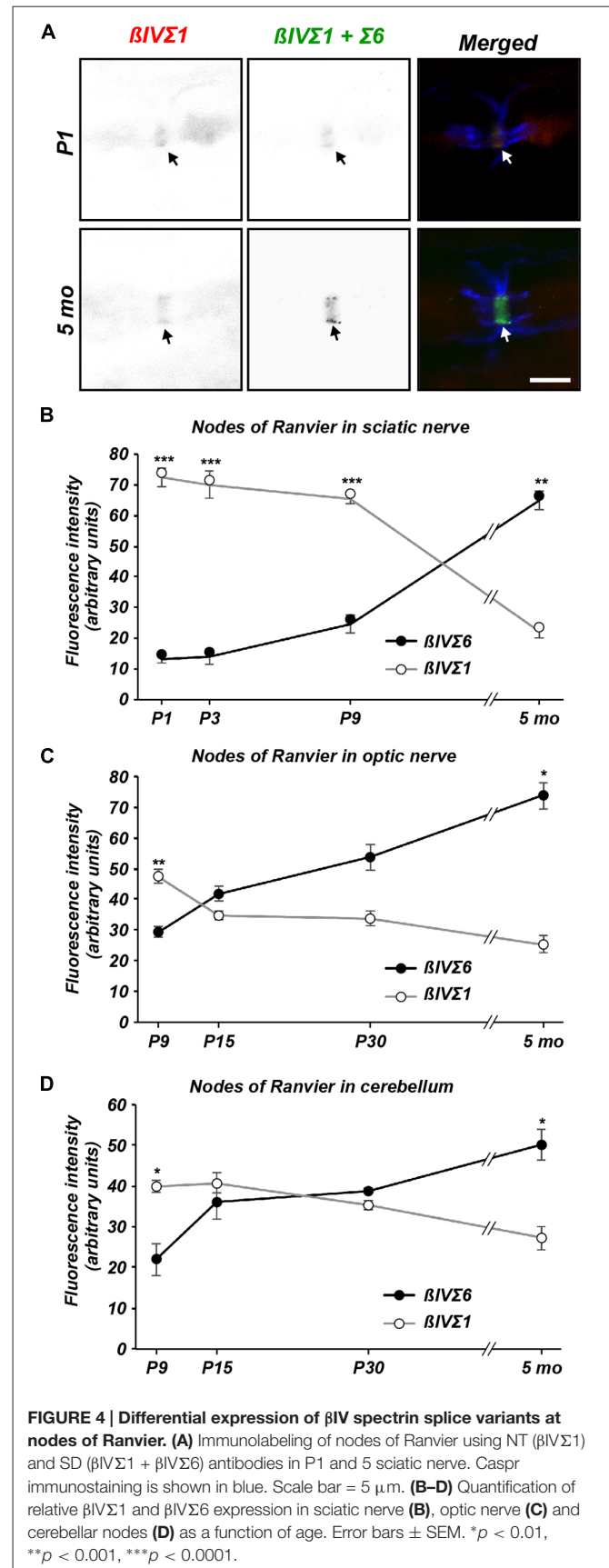
### DISCUSSION

$\beta$ IV spectrin is highly enriched at the AIS and nodes of Ranvier and functions to link membrane proteins to the actin-based

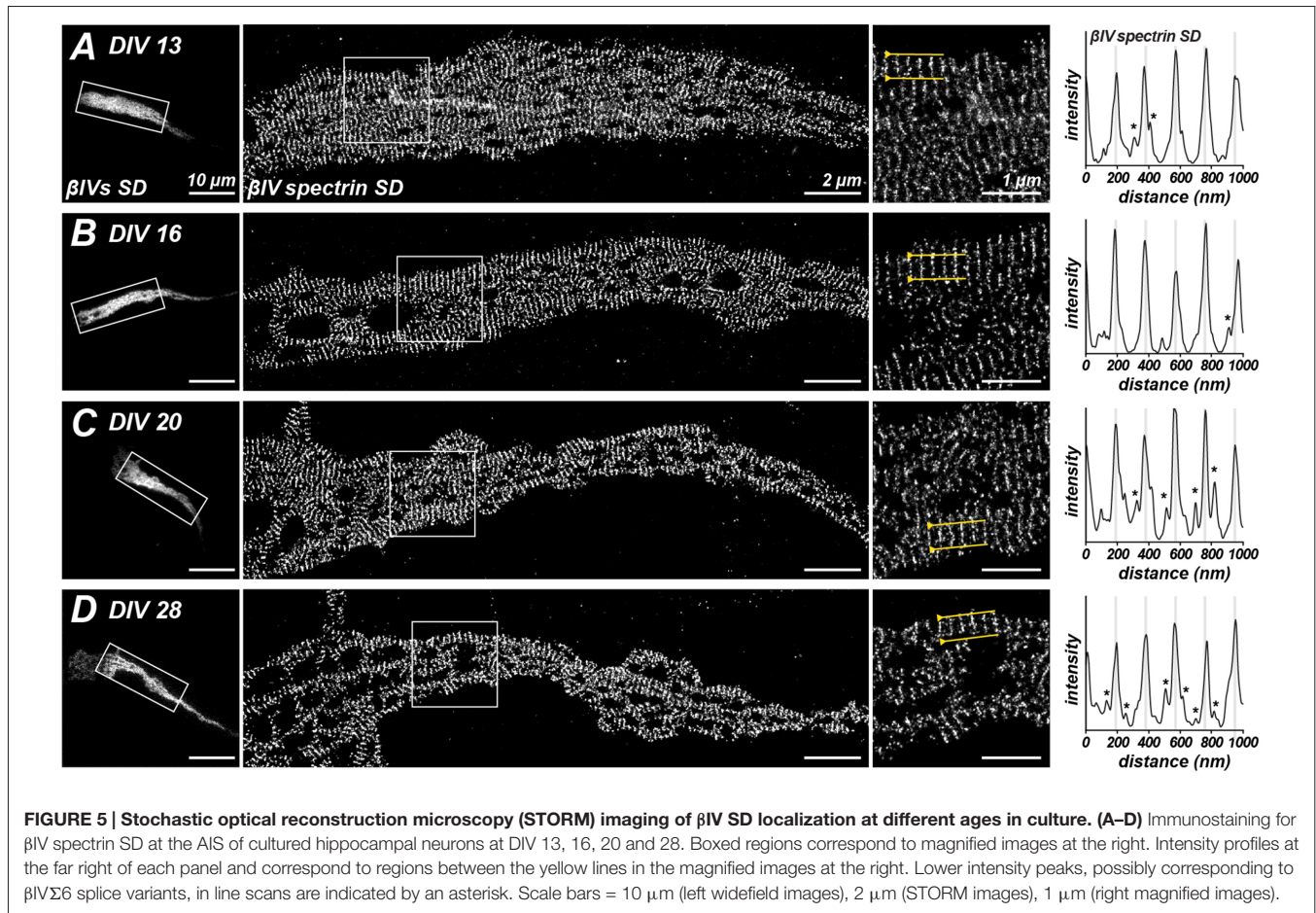


cytoskeleton through ankG (Berghs et al., 2000; Komada and Soriano, 2002; Yang et al., 2007). Mouse mutants of  $\beta$ IV spectrin have a myriad of nervous system abnormalities including disrupted nodes and AIS, hearing loss, ataxia, and even axon degeneration (Parkinson et al., 2001; Yang et al., 2004). Here, we showed that a shift from  $\beta$ IV $\Sigma$ 1 to  $\beta$ IV $\Sigma$ 6 occurs at the AIS and nodes of Ranvier during development, with  $\beta$ IV $\Sigma$ 1 being dominant in early development, but  $\beta$ IV $\Sigma$ 6 being dominant in mature neurons.

Spectrin  $\alpha$  and  $\beta$  subunits form antiparallel dimers that self-associate to form tetramers of  $\sim$ 190 nm in length, and that function as a submembranous scaffolding network anchored to actin filaments by  $\beta$  spectrins (Bennett and Healy, 2008). In axons, the spectrin-actin cytoskeleton forms a periodic network of circumferential actin rings along the inner cytoplasmic face of the axon membrane; these actin rings are evenly spaced along axonal shafts by spectrin tetramers. This network has been proposed to participate in both the polarization of the AIS membrane and to provide structural support to axons to withstand mechanical stresses (Xu et al., 2013). Despite the canonical view of spectrin tetramers, the composition of the AIS spectrin cytoskeleton remains an enigma since: (1) the







$\alpha$  spectrin subunit that partners with  $\beta$ IV spectrin has not yet been reported; and (2)  $\beta$ IV $\Sigma$ 6 lacks both the actin and  $\alpha$  spectrin-binding domains. Despite our observation that  $\beta$ IV $\Sigma$ 6 is the main splice variant in mature neurons, the length of spectrin tetramers, as measured using SD antibodies, remained constant at about 190 nm. In addition, intensity profiles along the AIS revealed secondary peaks in SD immunoreactivity that may correspond to spectrin tetramers that include a  $\beta$ IV $\Sigma$ 6 subunit. Thus, it is possible that a single AIS (or node) spectrin tetramer consists of both  $\beta$ IV $\Sigma$ 1 and  $\beta$ IV $\Sigma$ 6 subunits. While co-immunoprecipitation experiments using expression constructs in heterologous cells may be able to determine if these splice variants can function in the same tetramer, the extreme detergent insolubility of the AIS makes it difficult to confirm that these tetramers of mixed splice variants exist in the brain. It is possible that  $\beta$ IV spectrin splice variants can associate with  $\alpha$  spectrins through previously unidentified interacting domains present in both  $\beta$ IV $\Sigma$ 1 and  $\beta$ IV $\Sigma$ 6. Future studies of mice lacking  $\beta$ IV $\Sigma$ 1 using STORM might provide key insights into how the periodic spectrin cytoskeleton forms and is maintained, and how  $\beta$ IV $\Sigma$ 6 interacts with the actin cytoskeleton. Furthermore, it will be important to determine if  $\alpha$  spectrin functions at the AIS in concert with  $\beta$ IV spectrin splice variants.

Different  $\text{Na}^+$  channels occupy distinct subdomains of the AIS. In cortical pyramidal neurons, Nav1.2 and Nav1.6 are found in the proximal and distal regions of the AIS, respectively (Hu et al., 2009). Furthermore, during development Nav1.2 is first found at nodes of Ranvier, but then as neurons mature, Nav1.6 replaces these channels. The switch in the type of nodal  $\text{Na}^+$  channels correlates with the onset of myelination and the sustained expression of Nav1.6 depends on myelination. Although the distributions of  $\beta$ IV $\Sigma$ 1 and  $\beta$ IV $\Sigma$ 6 are similar in the proximal and distal regions of the AIS, the switch in  $\beta$ IV spectrin splice variants was also coincident with the onset of myelination. However, since the switch in splicing also occurs in cultured hippocampal neurons that lack myelin, the change in splice variant expression does not depend on myelination. Furthermore, the switch from Nav1.2 to Nav1.6 is unlikely to depend on the change in  $\beta$ IV spectrin splice variant expression since  $\text{Na}^+$  channel binding to ankG is both necessary and sufficient for their AIS and nodal localization (Garrido et al., 2003; Gasser et al., 2012).

Why is there an increase in the amount of  $\beta$ IV $\Sigma$ 6 expression at AIS and nodes with increasing age? Although our experiments cannot answer this, it is unlikely that replacing  $\beta$ IV $\Sigma$ 1 with



$\beta$ IV $\Sigma$ 6 reflects the recruitment of additional proteins to the AIS since all of the protein coding regions found in  $\beta$ IV $\Sigma$ 6 are also found in  $\beta$ IV $\Sigma$ 1. Instead, we speculate that a decrease in  $\beta$ IV $\Sigma$ 1 may reflect a maturation of the AIS and loss of some proteins that associated with the N-terminal half of  $\beta$ IV $\Sigma$ 1 during AIS assembly or establishment of the trafficking filter associated with the AIS.

In conclusion, using two kinds of anti- $\beta$ IV spectrin antibodies, NT for  $\beta$ IV $\Sigma$ 1 and SD for both  $\beta$ IV $\Sigma$ 1 and  $\beta$ IV $\Sigma$ 6 splice variants, we found a switch in the expression levels and localization of these splice variants at the AIS and nodes of Ranvier during development. Additional experiments will be required to determine the role this switch has for nervous system function.

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## AUTHOR CONTRIBUTIONS

TY and SRS performed immunoblots, immunostaining and quantification of signals. CL performed STORM imaging. MCS performed RT-qPCR. MNR and MCS conceived and directed the project. All authors contributed to writing and revising of the final manuscript.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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