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Caspr2, a New Member of the Neurexin Superfamily, Is Localized at the Juxtaparanodes of Myelinated Axons and Associates with K^+ Channels

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Summary

Rapid conduction in myelinated axons depends on the generation of specialized subcellular domains to which different sets of ion channels are localized. Here, we describe the identification of Caspr2, a mammalian homolog of *Drosophila* Neurexin IV (Nrx-IV), and show that this neurexin-like protein and the closely related molecule Caspr/Paranodin demarcate distinct subdomains in myelinated axons. While contactin-associated protein (Caspr) is present at the paranodal junctions, Caspr2 is precisely colocalized with Shaker-like K^+ channels in the juxtaparanodal region. We further show that Caspr2 specifically associates with Kv1.1, Kv1.2, and their Kvβ2 subunit. This association involves the C-terminal sequence of Caspr2, which contains a putative PDZ binding site. These results suggest a role for Caspr family members in the local differentiation of the axon into distinct functional subdomains.

Introduction

The axons of myelinated nerves in the adult nervous system are subdivided into several distinct structural and functional domains that each differ in their molecular composition (Salzer, 1997; Waxman, 1997). The generation of these specialized subcellular structures is essential for the efficient and rapid propagation of action potential via saltatory conduction. Several distinct subdomains could be found in the axonal membrane, the nodes of Ranvier, the paranodes, and the juxtaparanodes. The nodes of Ranvier are characterized by a high concentration of voltage-gated Na^+ channels, which enables the regeneration of the action potential (Waxman and Ritchie, 1993). Several other proteins were shown to be localized to the axolemma at the nodes, including Na^+/K^+ ATPases, the spectrin-binding protein ankyrin, and the cell adhesion proteins NrCAM and Neurofascin (Kordeli et al., 1995; Davis et al., 1996; Scherer, 1996). Junctions that are formed between the axon and the myelinating cell (called the paranodal junctions) border the nodes of Ranvier. In this region, the compact myelin lamellae open up into a chain of cytoplasmic loops that form a series of septate-like junctions with the axon (Rosenbluth, 1995). The paranodal region is thought to serve several functions: to anchor the myelin to the axon, to form a partial barrier that isolates the periaxonal space under the myelin from the electrical activity at the nodes, and to physically demarcate boundaries that limit the lateral diffusion of membrane components, thereby confining them to the node. A third specialized region in myelinated axons is defined as the juxtaparanode. This region, often referred to as the paranodal main segment, is located in a short zone just beyond the innermost paranodal junctions, separating them from the internodes that lie beneath the compact myelin sheaths (Berthold and Rydmark, 1995). In large fibers in the PNS, this region contributes to the axon–Schwann cell network, a structure of thin axonal processes that are enclosed by protrusions of the Schwann cell cytoplasmic layer (Berthold and Rydmark, 1995). This complex network is implicated in axonal transport and in the lysosyme-mediated degradation of transported material (Gatzinsky, 1996; Zimmermann, 1996). Although no prominent network is present in the CNS, some invasion of the axoplasm by the inner cytoplasmic loop occurs at this site (Ellisman et al., 1984). The juxtaparanodal region is characterized by the presence of heteromultimers of the Shaker-like K^+ channel α subunits Kv1.1 and Kv1.2, and the cytoplasmic Kvβ2 subunit (Wang et al., 1993; Rhodes et al., 1997; Rasband et al., 1998). The precise localization of voltage-activated K^+ channels to this region may stabilize conduction and help to maintain the internodal resting potential (Zhou et al., 1998; Vabnick et al., 1999).

Contactin-associated protein (Caspr; also known as Paranodin) is a member of the neurexin superfamily, a group of transmembrane proteins that mediate cell–cell interactions in the nervous system (Baumgartner et al., 1996; Menegoz et al., 1997; Peles et al., 1997; Bellen et al., 1998; Missler and Südhof, 1998). It was originally identified in a complex with contactin that binds to the receptor protein tyrosine phosphatase β (RPTPβ; Peles et al., 1995, 1997) and as a major lectin-binding protein from rat brain (Menegoz et al., 1997). Caspr is concentrated at the paranode of Ranvier in the septate-like junctions that are formed between axons and the terminal loops of oligodendrocytes and myelinating Schwann cells (Einheber et al., 1997; Menegoz et al., 1997). Both the localization of Caspr and the development of the septate junctions occur with the maturation of the myelinated fiber, suggesting that Caspr may be an essential component of the paranodal junction (Tao-Cheng and Rosenbluth, 1982; Einheber et al., 1997). The notion that

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Caspr plays a role in the generation and integrity of the paranodal junctions is also supported by studies in *Drosophila* demonstrating that the Caspr-related Neurexin IV (Nrx-IV) is an essential component of the ectodermally derived pleated septate junctions (Baumgartner et al., 1996). Nrx-IV mutants are devoid of septate junctions between glial cells, resulting in the breakdown of the blood–nerve barrier. These mutants lack the transverse septate characteristic of these junctions, which are structurally similar to the paranodal junctions (Baumgartner et al., 1996).

Although Caspr and the related *Drosophila* Nrx-IV have a similar primary structure, they differ in their cytoplasmic tail. Caspr contains a proline-rich sequence, while *Drosophila* Nrx-IV has a shorter cytoplasmic region containing a binding site for PDZ domains (Bellen et al., 1998). This structural difference suggested the existence of other members of this group in vertebrates. Here, we describe the identification of Caspr2, a new member of this family, as a component of the juxtaparanodal region in myelinated axons.

Results

Searching the databases for Caspr homologs revealed the presence of several *caspr*-related expressed sequence tags (ESTs) from various nervous system sources. One of these ESTs (R13972) was sequenced and used as a specific probe to clone the full-length human and mouse *caspr2* cDNAs from brain and spinal cord cDNA libraries (Figure 1A). The structural organization of the encoded Caspr2 polypeptide is very similar to that of Caspr and Nrx-IV, all having the hallmarks of type I transmembrane proteins. The extracellular region of Caspr2 is a mosaic of domains, including discoidin/neuropilin and fibrinogen-like domains, two epidermal growth factor (EGF) repeats, and four domains similar to a region in laminin A, referred to as the G domain (Figure 1C). The extracellular region of Caspr2 contains 12 potential N-linked glycosylation sites, compared with 17 and 9 sites in Caspr and Nrx-IV, respectively. Human *caspr2* and *caspr* transcripts have open reading frames that encode for 1331 and 1384 amino acids, respectively, and exhibit 45% identity at the amino acid level (Figure 1A). An alignment of human Caspr proteins with the amino acid sequence of *Drosophila* Nrx-IV revealed that Caspr2 is more related (34% identity) to Nrx-IV than is Caspr (29% identity). Like Nrx-IV, Caspr2 lacks the proline–glycine–tyrosine repeats that are found near the transmembrane domain of Caspr. The major difference between Caspr2 and Caspr is found in the cytoplasmic domain. While both share a protein 4.1 binding sequence at the juxtamembrane region, they diverge thereafter. As shown in Figure 1B, the intracellular region of Caspr2 is more similar to glycophorin C (65% identity) and Nrx-IV (37% identity) than to Caspr (29% identity). Caspr2, Nrx-IV, and all the neurexins each contain a short amino acid sequence at their C terminus, which serves as a binding site for type II PDZ domains (Bellen et al., 1998; Missler and Südhof, 1998; Bhat et al., 1999). This consensus sequence is not found in Caspr (Peles et al., 1997). Altogether, these sequence similarities indicate that Caspr2 is most likely the mammalian ortholog of *Drosophila* Nrx-IV.

Caspr2 Is Predominantly Expressed in the Nervous System

Analysis of the expression of *caspr2* mRNA in various human tissues by Northern blots is presented in Figure 2A. Two transcripts, of 9 and 10 kb, were found in the brain, while only the 9 kb transcript was detected in the spinal cord. Low but detectable levels of *caspr2* mRNA were also detected in the ovary and prostate but not in other human tissues (Figure 2A). We next compared the expression of the two *caspr* transcripts in different regions of the adult human CNS (Figure 2B). In spinal cord and corpus callosum, only the 9 kb *caspr2* transcript was found. This was also the predominant transcript in the medulla, substantia nigra, and caudate nucleus. All other regions examined expressed similar levels of the 9 and 10 kb *caspr2* transcripts. The distribution of *caspr* mRNA was similar to that observed for Caspr2, with the exception of the corpus callosum and the spinal cord, where no *caspr* mRNA was detected (Figure 2B; Peles et al., 1997). This difference in the corpus callosum may reflect the expression of Caspr2 in both neurons and glial cells in contrast to the restricted expression of Caspr in neurons (see below). These analyses demonstrated that like Caspr, Caspr2 is predominantly expressed in the nervous system.

Distribution of Caspr2 Protein in Rat Brain

To study the expression of Caspr2 protein, we have generated polyclonal antibodies against its intracellular region. These antibodies specifically recognized a protein with an apparent MW of 180 kDa in lysates from HEK293 cells transfected with a vector that directed the expression of Caspr2 (Figure 3A). No signal was obtained from nontransfected cells or cells transfected with the related *caspr* cDNA. In addition, antibodies to Caspr did not react with Caspr2, indicating that the two Caspr proteins could specifically be detected using our antibodies. Anti-Caspr2 antibodies also recognized the endogenous protein when immunoprecipitation and immunoblotting were performed on solubilized adult rat brain membranes (Figure 3B). In addition, these antibodies specifically stained Caspr2-transfected HEK293 cells, confirming the membrane localization of Caspr2 protein as predicted by its deduced amino acid sequence (data not shown). Thus, anti-Caspr2 antibodies do not cross-react with the related Caspr protein and specifically recognize a protein of the same size from both rat brain and Caspr2-transfected cells. In addition to the 180 kDa protein, anti-Caspr2 antibody recognized in rat brains, during embryonic stages, a protein of 130 kDa that disappeared during the first postnatal week (Figure 3C). The 180 kDa Caspr2 protein was first detected as early as embryonic day 15 (E15), but its expression continued to increase as development progressed, reaching maximal levels in the adult. In contrast, Caspr was seen primarily in postnatal stages, reaching its highest levels in the adult. This pattern of expression is in agreement with earlier studies demonstrating an increase in Caspr levels with the onset of myelination (Menegoz et al., 1997).

We next examined the distribution of Caspr2 protein in sections of adult rat brain using affinity-purified antibodies. Throughout the brain, Caspr2 was detected primarily in neurons (Figure 4). No signal was obtained

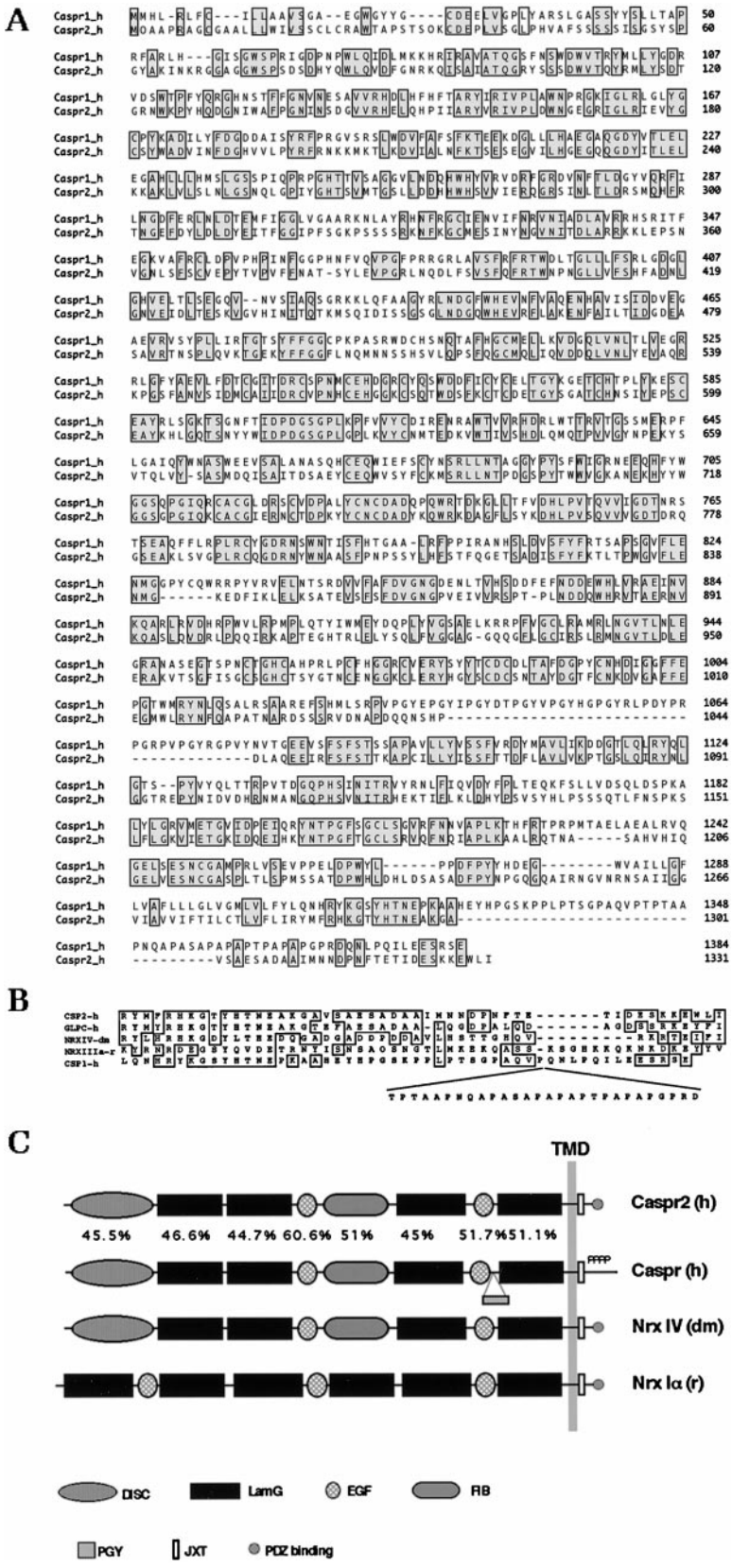

Figure 1. Primary Structure and Domain Organization of Caspr2

(A) Deduced amino acid sequence of human Caspr2 compared with Caspr. Identical amino acids are boxed.

(B) Alignment of the cytoplasmic tail of human Caspr2 (CSP2-h), human glycophorin C (GLPC-h), *Drosophila* Nrx-IV (NRXIV-dm), rat neurexin IIIα (NRXIIIα-r), and human Caspr (CSP1-h). The proline- and alanine-rich sequence shown below is found only in the cytoplasmic domain of Caspr and not in the other proteins.

(C) Structural organization of the Caspr proteins. Schematic comparison of human Caspr proteins with *Drosophila* Nrx-IV and rat neurexin Iα. A discoidin-like domain (DISC) and a region similar to fibrinogen (FIB) are found in Caspr, Nrx-IV, and Caspr2 but not in the related mammalian neurexins. A repeat region of proline, glycine, and tyrosine residues (PGY) is found only in Caspr. All members of the Caspr (NCP) and neurexin families share other motifs, including laminin G domains (LamG) and EGF repeats. They also share a conserved juxtamembrane sequence (JXT) in their cytoplasmic tail but diverge thereafter. Caspr contains a unique, proline-rich region, whereas Caspr2, Nrx-IV, and the neurexins contain a C-terminal binding site for PDZ domains.

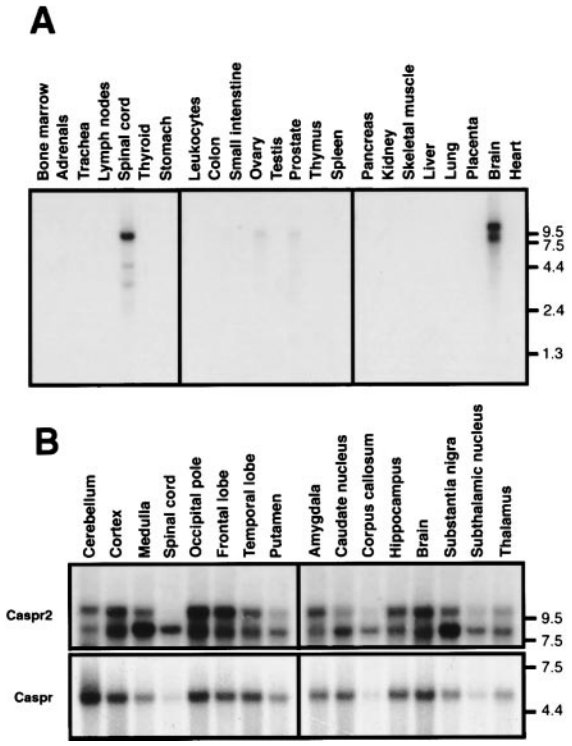

Figure 2. Expression of *caspr2* mRNA in Different Tissues

(A) Northern blots containing mRNA from various tissues were hybridized with a Caspr2-specific probe. The autoradiogram is shown along with MW markers on the right.

(B) Expression of *caspr2* (upper panel) and *caspr* (lower panel) mRNA in different regions in the CNS.

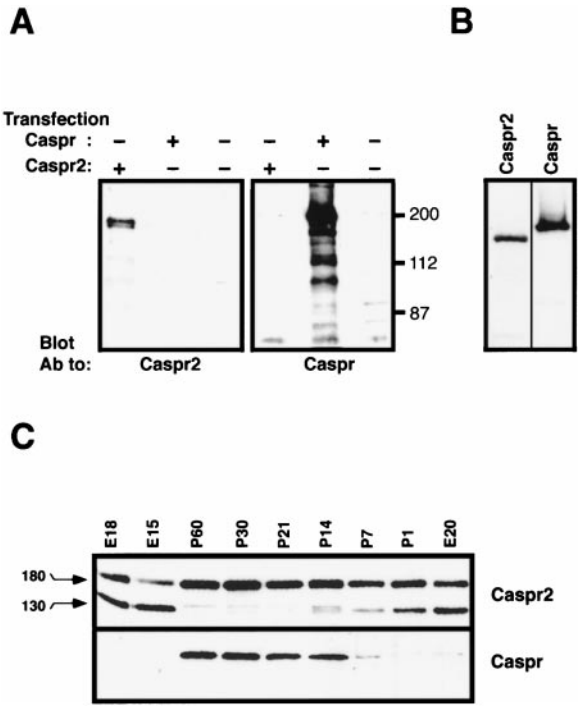

Figure 3. Caspr2 Encodes for a 180 kDa Protein

(A) COS7 cells were transfected with either Caspr or Caspr2 expression vectors, as labeled (−/+). Cell lysates were subjected to immunoprecipitation and immunoblot analysis using anti-Caspr2 (left panel) or Caspr (right panel) antibodies. MW markers, in kilodaltons, are shown on the right.

(B) Solubilized rat brain membranes were subjected to immunoprecipitation and Western blot analysis with specific antibodies to Caspr2 or Caspr, as indicated. The difference in the apparent MW between the two casprs is clearly observed.

(C) Developmental expression of Caspr2 and Caspr. Equal amounts of rat brain membrane proteins prepared from different embryonic and postnatal days were separated on SDS gels and blotted with the indicated antibodies. Note that anti-Caspr2 antibody recognized two proteins of the apparent MW of 180 and 130 kDa during different developmental stages. Both forms were detected already in E15 brains, but while the expression of the 180 kDa protein increases during development to its maximal levels in the adult, the expression of the 130 kDa protein decreased postnatally and was undetectable in the adult.

when the antibodies were preincubated with Caspr2 intracellular domain (data not shown). Specific staining of cell bodies and dendrites was observed in pyramidal cells in the CA3 region of the hippocampus (stratum radiatum), as well as in the subicular complex, the amygdala, and the cortex. In the latter, Caspr2 immunoreactivity was observed in neurons in the fifth pyramidal layer, which extended long dendrites toward the second cortical layer (Figure 4C). In the dentate gyrus, Caspr2 labeling was confined to triangular and multipolar mossy cells in the polymorphic cell layer, as well as to some widely dispersed cell bodies throughout the granule cell layer (Figure 4D). In addition, diffuse staining was detected in the striatum, the globus pallidus, and the internal capsule (data not shown). Strong labeling of the granular cell layer and periglomerular cells surrounding the glomeruli was detected in the olfactory bulb (Figure 4E; data not shown). Caspr2 immunoreactivity was also found in higher olfactory centers, including the olfactory tubercle, the amygdaloid complex, the lateral entorhinal area, and the piriform cortex (data not shown). In addition to neuronal staining, Caspr2 antibodies labeled unicellular rows of cells, presumably interfascicular oligodendrocytes, in the cerebellar peduncle and the fimbria (Figure 4F; data not shown). These rows of cells are part of the macroglial framework, which consists of oligodendrocytes and astrocytes aligned along the longitudinal axonal axis of the tracts (Suzuki and Raisman, 1994). In regions of the brain containing myelinated fibers, Caspr2 appeared as intermittent pairs of lines, reminiscent of paranodal Caspr staining but with a longer distance between stained regions (compare Figures 4G and 4H). Higher magnification analysis revealed that similar staining patterns were present in all regions of the CNS examined (data not shown). These analyses demonstrated that Caspr2 is differentially expressed in distinct neuronal structures, including the soma and dendrites, and in specific short-segmented pairs along myelinated axons.

Caspr Family Members Demarcate Different Domains in Myelinated Nerves

The intermittent staining along axons suggested that Caspr2 could be expressed in the juxtaparanodal region, close to the nodes of Ranvier. To investigate this possibility, we double labeled sections of rat optic nerve with antibodies for Caspr2 and Caspr. As shown in Figure 5,

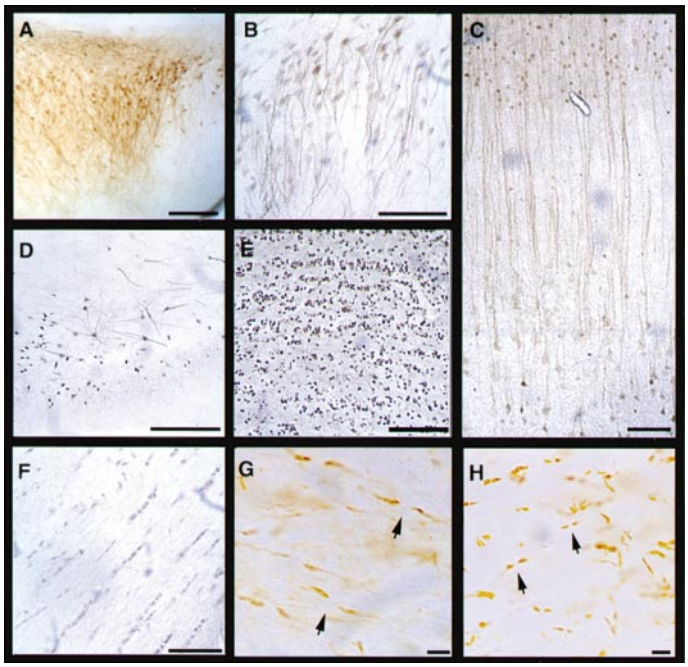

Figure 4. Immunolocalization of Caspr2 Protein in Adult Rat Brain

(A and B) Immunoperoxidase staining was performed on longitudinal 35–50 μm floating sections of rat brain using Caspr2 antibodies. Expression of Caspr2 protein was detected in cell bodies and dendrites in the amygdala (A) and in pyramidal cells in the CA3 region of the hippocampus (B).

(C) In the cerebral cortex, there is a prominent Caspr2 staining in the cell bodies and dendrites of neurons in the fifth pyramidal layer that extended their long dendrites toward the second cortical layer.

(D) Triangular and multipolar cells in the polymorphic cell layer in the dentate gyrus exhibit intense staining of the cell bodies and dendritic processes; there is also labeling of cell bodies in the granular cell layer.

(E) Section of the olfactory bulb showing intense labeling of Caspr2 in granular cells.

(F) In the cerebellar peduncle, strong Caspr2 immunoreactivity is detected in unicellular rows of interfascicular oligodendrocytes, which aligned along the longitudinal axonal axis of the tracts. These cells are distinctly negative when stained with Caspr antibodies (data not shown).

(G) In the cerebellar white matter, Caspr2 staining appears as intermittent pairs of short segments along the axons. Similar staining was observed in white matter throughout the brain. Note that this staining pattern is reminiscent of the paranodal Caspr staining (shown for comparison in [H]) but has a longer distance between the stained regions.

Scale bar, 200 μm (A–F) and 20 μm (G and H).

Caspr2 expression was confined to short segments that bordered the paranodal Caspr staining. However, in contrast to Caspr, which was found in all of the paranodes in the section, strong juxtaparanodal staining with Caspr2 antibody was mainly detected in large caliber axons. At the remaining sites, Caspr2 staining was weak or in some cases undetectable. In large caliber fibers, the axons are constricted at the paranodal region, with the diameter reduced by up to 50% of the internodal value (Hildebrand et al., 1993). In these fibers, Caspr2 is expressed in the juxtaparanodal region, just before the point at which the axon caliber is reduced. The nodes of Ranvier themselves could be visualized using an antibody against voltage-gated Na^+ channels. Staining for Caspr2 and Na^+ channels resulted in the appearance of two entirely separate zones corresponding to the nodes and the distal juxtaparanodal regions (Figure 5D). In contrast, antibodies to Caspr labeled the gap between Caspr2 and Na^+ channels, which represents the paranodes (Figure 5E). Triple staining for Na^+ channels, Caspr2, and Caspr clearly demonstrated that the Caspr proteins are found in nonoverlapping, distinct domains near the nodes of Ranvier (Figure 5F). Together, these data demonstrate that while Caspr is found in the paranodes, Caspr2 is expressed at the juxtaparanodal region.

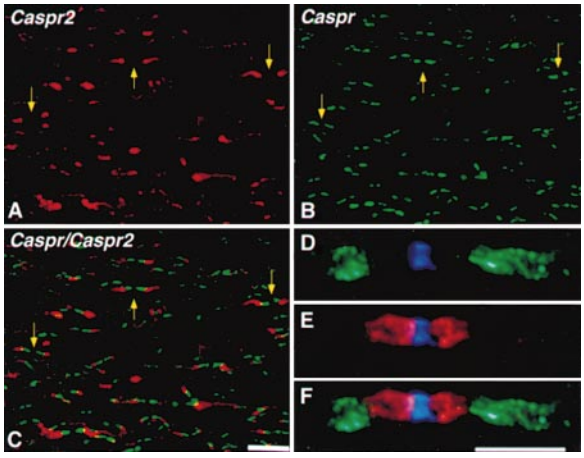

Figure 5. Members of the Caspr Family Are Localized to Distinct Axonal Domains

(A–C) Double labeling of longitudinal sections of rat optic nerve using antibodies to Caspr2 (red) and Caspr (green), or the combined image as indicated on the top of each panel. Arrows highlight the location of several representative nodal regions.

(D–F) Sections of rat optic nerve were triple labeled with antibodies against Na^+ channels (blue), Caspr (red), and Caspr2 (green). The relative localization of Na^+ channels to Caspr2 (D) or Caspr (E), or the combined image (F), is shown.

Scale bar, 10 μm (A–C) and 5 μm (D and F).

Caspr2 Is Found in the Juxtaparanodal Axolemma

To determine whether Caspr2 is found in the axonal and/or the oligodendrocyte membrane, we examined the distribution of the protein by immunoelectron microscopy. Figure 6 shows micrographs of Caspr2 antibody staining of myelinated fibers in the arbor vitae of the cerebellum using the immunoperoxidase technique. Dense Caspr2 labeling was detected in the juxtaparanodal region and was associated with the axonal membrane. In addition to expression at the inner surface of the axonal membrane, flocculent staining was visible

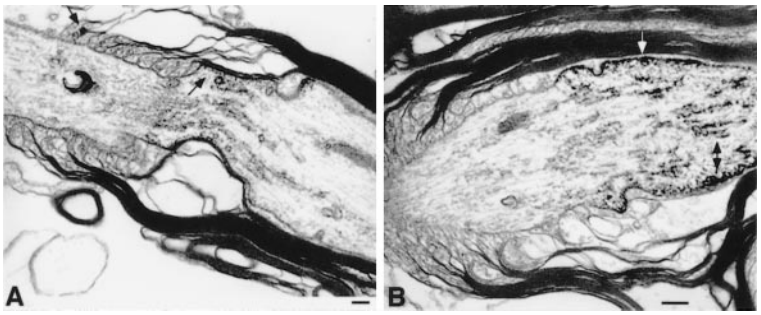

Figure 6. Immunoelectron Microscopic Localization of Caspr2 in Myelinated Nerves

Immunoperoxidase labeling of Caspr2 in longitudinal sections of nodal regions of cerebellar myelinated fibers (A and B). Arrows mark dense labeling of the inner surface of the axonal membrane in the juxtaparanodes, as well as a flocculent cytoplasmic staining within the axon in this region. Note that no labeling was observed in the axonal paranodes or internode (A). In a few fibers, labeling was observed in some of the paranodal loops close to the nodes (arrow, [A]). Scale bar, 0.5 μm.

throughout the axoplasm of the juxtaparanodal region. This flocculent label was specific to the juxtaparanodal region and was greatly reduced or completely absent under the paranodal loops or compact myelin. A similar staining pattern was previously reported for the Kv1.2 K^+ channel (Wang et al., 1993). In addition to the axon, we have occasionally detected Caspr2 in the outermost paranodal loops (Figure 6A). Similar analyses showed that Caspr2 was localized to the axonal membrane of the juxtaparanodal region in the optic nerve (data not shown). Thus, the expression of Caspr2 in myelinated nerves is mostly confined to the axon at the juxtaparanodal region and to some isolated paranodal loops.

Colocalization and Association of Caspr2 with K^+ Channels

The Shaker-type K^+ channel α subunits Kv1.1 and Kv1.2, and their cytoplasmic subunit Kvβ2, are known to be localized to the juxtaparanodes (Wang et al., 1993; Rhodes et al., 1997; Rasband et al., 1998; Vabnick et al., 1999). We have therefore compared the localization of Caspr2 with that of Kv1.2 in sections of adult rat optic and sciatic nerves. Double-labeling experiments revealed that both proteins are localized to the same axonal domain (Figure 7). Similar staining was obtained in sciatic nerves. However, in the PNS, Caspr2 was found only in about 15% of the juxtaparanodal sites. In some preparations, the expression of Caspr2 was found to be more confined to the paranodal border than that of K^+ channels, which was more diffuse and extended further toward the internode (Figure 7E). As with Caspr2 staining, Kv1.2 immunoreactivity was more prominent in large fibers. During development of the optic nerve, Caspr2 and Kv1.2 appeared simultaneously in the juxtaparanodes and could be detected at just a few sites at postnatal day 12 (P12), and with increasing frequency later (data not shown).

These observations prompted us to investigate whether Caspr2 could be found in a complex with K^+ channels. We therefore performed coimmunoprecipitation experiments using specific antibodies to several different K^+ channel subunits. When lysates from rat brain membranes were subjected to immunoprecipitation with anti-Kv1.2 or anti-Kvβ2 antibodies, Caspr2 was detected in these immunocomplexes (Figure 8A). In contrast, Caspr2 was not coimmunoprecipitated with an antibody against the Kv2.1 subunit. In addition, Caspr2 antibodies specifically precipitate Kv1.2 and Kvβ2 but not Kv2.1. No signal was obtained when Caspr2 preimmune serum was used. Furthermore, absorption of Caspr2-specific antibodies using a GST–Caspr2 from the serum resulted in a proportional decrease in the amount of the Kv1.2 that was detected in the immunocomplexes (Figure 8A). Together with the immunocytochemical data, these experiments demonstrate that in rat brain, Caspr2 is found in a physical complex with Shaker-like K^+ channels.

The interaction between Caspr2 and K^+ channels is indirect and is probably mediated by an additional protein, since no stable complex of these proteins could be detected in COS7 or HEK293 cells expressing Caspr2 and Kv1.2 or Kvβ2 (data not shown). The presence of a PDZ domain binding site in the C-terminal tails of Caspr2, Kv1.1, and Kv1.2 raises the question of whether these proteins might be associated in a complex through a common PDZ domain–containing protein. To address this possibility, we have asked whether the C-terminal region of Caspr2 could precipitate Kv1.2. As depicted in Figure 8B, the C-terminal peptide of Caspr2 (position 1317–1331) immobilized to agarose specifically pulled down Kv1.2 from rat brain membrane lysates. In contrast, Kv1.2 was not detected in these blots when a

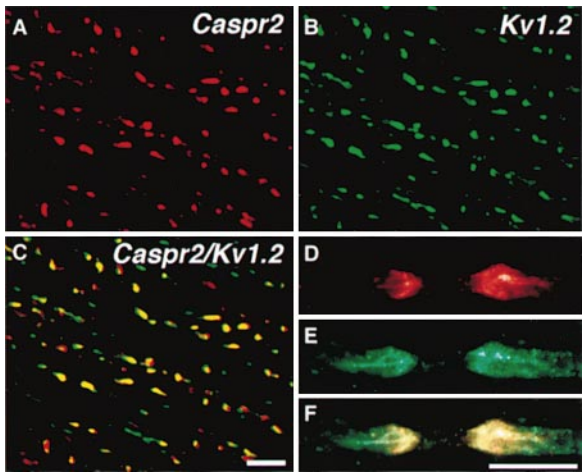

Figure 7. Localization of Caspr2 and the Kv1.2 K^+ Channel to the Juxtaparanodes

Longitudinal sections of rat optic nerve were double labeled with antibody to Caspr2 (red) and Kv1.2 (green), as indicated.
(A–C) Deconvoluted digital images (A and B) detected using a single filter. The dual filter image of this experiment is shown in (C).
(D–F) High magnification of a single nodal region in sciatic nerve double labeled for Caspr2 (red) and Kv1.2 (green). Single filter (D and E) and double (F) images are shown.
Scale bar, 10 μm (A–C) and 5 μm (D and F).

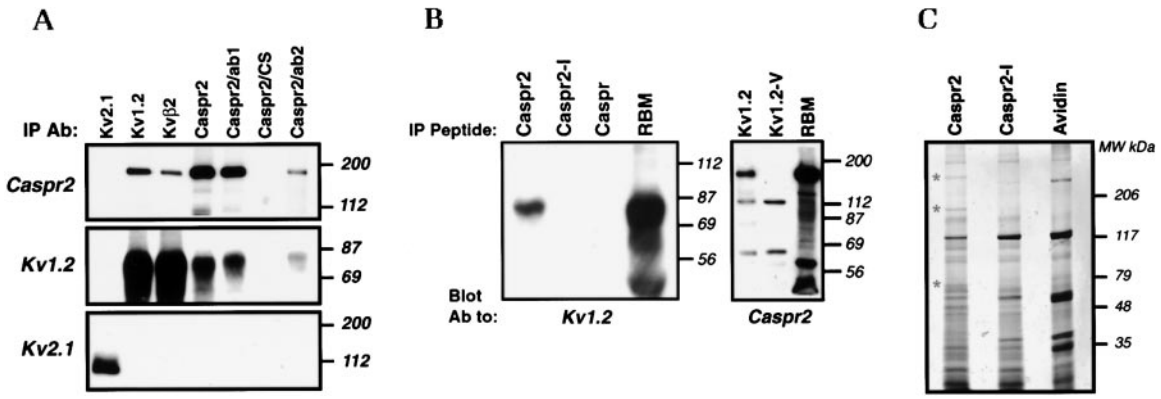

Figure 8. Association of Caspr2 with K^+ Channels

(A) Rat brain membrane lysates were immunoprecipitated with either antibodies to Caspr2; antibodies to Caspr2 that were preabsorbed on GST–Caspr (Caspr2/ab1); or GST–Caspr2 (Caspr2/ab2) fusion proteins, antibodies to different K^+ channel subunits, or control preimmune serum (Caspr2/CS), as indicated at the top (IP Ab). Proteins were separated on SDS gels and immunoblotted with antibodies against Caspr2, Kv1.2, or Kv2.1, as indicated on the left of each panel. MW markers are shown on the right in kilodaltons.

(B) Agarose-bound peptides derived from the C-terminal tail of Caspr2 (Caspr2), Caspr (Caspr), a Caspr2 peptide lacking the last isoleucine (Caspr2-I), Kv1.2 peptide (Kv1.2), or a Kv1.2 peptide lacking the last valine (Kv1.2-V) were incubated with rat brain membrane lysates as indicated (IP Peptide). Bound proteins were separated on SDS gel and immunoblotted with either an antibody to Kv1.2 (left panel) or an antibody to Caspr2 (right panel). Rat brain membrane proteins (RBM) were use as a positive control for the location of Caspr2 and Kv1.2 in the gel. MW markers are shown on the right in kilodaltons.

(C) Silver staining of a pull-down experiment from rat brain membrane lysate using the C-terminal tail of Caspr2 (Caspr2), a Caspr2 peptide lacking the last isoleucine (Caspr2-I), or avidin-agarose as control (avidin). Three proteins that were specifically pulled down by Caspr2 peptide are marked with an asterisk on the left. The location of MW markers is shown on the right in kilodaltons.

peptide derived from the C-terminal tail of Caspr (position 1373–1384) or a Caspr2 peptide lacking the last amino acid (isoleucine at position 1331; Caspr2-I) was used. Moreover, a C-terminal peptide of Kv1.2 (position 488–489 of rat Kv1.2), but not a peptide lacking the last valine, which is known to be essential for the association of this channel with PDZ domain–containing proteins (reviewed by Sheng, 1997), pulled down Caspr2 from rat brain membrane lysates (Figure 8B). We have estimated the fraction of Kv1.2 that could be precipitated with Caspr2 to be $\sim<$0.1% of the total Kv1.2 protein detected in the lysate. This low yield possibly reflects the relative lower quantity of Kv1.2 found in the juxtaparanodes compared with the whole brain and may indicate that the juxtaparanodes are the primary site of association between Caspr2 and K^+ channels. Finally, silver staining revealed the presence of three proteins, with the apparent MW of 240, 160, and 70 kDa, that specifically pulled down with the normal but not the mutant (missing the last isoleucine) Caspr2 C-terminal peptide (Figure 8C). It is worth noting that the 70 kDa protein has a similar apparent MW of Kv1.2; however, additional protein sequencing would be required to determine whether this is the case, as well as to reveal the identity of the 240 and 160 kDa proteins. These results indicate that in both Caspr2 and Kv1.2, the C-terminal region, which contains the PDZ binding site, is sufficient for their association.

Discussion

Caspr2 Is a Novel Member of the Neurexin Superfamily

The neurexin superfamily consists of a growing list of both secreted and transmembrane proteins (Bellen et al., 1998; Missler and Südhof, 1998). These include the neurexins themselves, which are encoded by three different genes (Ushkaryov et al., 1992, 1994; Ushkaryov and Südhof, 1993); the NCP subgroup (for Nrx-IV/Caspr/Paranodin; Bellen et al., 1998), which consists of Caspr/Paranodin (Menegoz et al., 1997; Peles et al., 1997); Nrx-IV and Caspr2 (Baumgartner et al., 1996; this work); and axotactin (Yuan and Ganetzky, 1999). Different structural features distinguish members of this superfamily. Caspr2, as the other NCPs, is distinguished from the neurexins by the presence of a discoidin/neuropilin homology domain and a novel fibrinogen-like region.

The extracellular region of several members of the neurexin superfamily mediates protein–protein interactions. The neurexins bind to various ligands, including neuroligins (Ichtchenko et al., 1995), neurexophilins (Petrenko et al., 1996), and α-latrotoxin (Sugita et al., 1999), while Caspr binds to the glycosylphosphatidylinositol-linked cell adhesion molecule contactin (Peles et al., 1997). In contrast, we have found that Caspr2 is not associated with contactin in rat brain (S. P. and E. P., unpublished data). Thus, despite their structural similarity, the respective extracellular regions of the Caspr proteins enable their interactions with different ligands.

Intracellularly, Caspr2 shares with all other transmembrane members of the neurexin superfamily a short cytoplasmic domain that contains a juxtamembrane binding site for members of the protein 4.1 family (Littleton et al., 1997; Menegoz et al., 1997) and (with the exception of Caspr) a C-terminal tail that binds PDZ domain–containing proteins (Butz et al., 1998; Bhat et al., 1999). Based on the structural similarity of Caspr2 and Nrx-IV, we suggest that the former is the vertebrate homolog of *Drosophila*'s Nrx-IV. However, it should be noted that the vertebrate NCP family may consist of at least three different genes. In humans, *caspr* is located on chromosome 17q21 (Peles et al., 1997), while the *caspr2* gene

was assigned by sequence-tagged site to chromosome 7q35–36 (SHGC-31280, between D7S688 and D7S505 in genemap98 at the National Center for Biotechnology Information). Partial sequence of the potential third member of this family (*caspr3*) can be found in the EST databases (see, for example, GB: AI148152). Given that the latter is found in various glial-derived tumor cell lines (I. Spiegel and E. P., unpublished data), we cannot exclude the possibility that it is the human ortholog of the invertebrate Nrx-IV. Both the neurexins and members of the NCP family mediate cell–cell interactions. However, while the neurexins are found in presynaptic sites and mediate interactions between neurons (Ushkaryov et al., 1992; Irie et al., 1997; Missler and Südhof, 1998), the NCP members are thought to mediate neuron–glia and, in invertebrates, glia–glia interactions (Baumgartner et al., 1996; Einheber et al., 1997; Menegoz et al., 1997; Peles et al., 1997). The localization of Caspr2 in the juxtaparanodal region of myelinated nerves is consistent with the proposed role of the NCP family in mediating neuron–glia interactions.

Members of the Caspr Family Demarcate Distinct Domains in Myelinated Axons

Proper conduction in myelinated nerve fibers depends on the clustering of ion channels to specific membrane domains in the axon (Salzer, 1997; Waxman, 1997). Ultrastructural studies showed that the axolemma is a highly specialized structure having different characteristics at the nodes, paranodes, juxtaparanodes, and internodes (Rosenbluth, 1984). Freeze-fracture replicas show clear differences in the size and organization of particles that are present in the juxtaparanodal region in comparison with the nodes and paranodes (Tao-Cheng and Rosenbluth, 1984). Moreover, the distribution of these particles was found to vary along the juxtaparanodal region, with a higher concentration near the paranode/juxtaparanode border, which gradually diminished in the direction of the internodes. The bilateral symmetric accumulation of Caspr2 and K^+ channels in the juxtaparanodal region, as shown here, correlates well with the distribution of these particles.

The localization of the Caspr proteins to distinct domains could be controlled by interactions with extracellular ligands, as well as with intracellular proteins within the axon. Candidate axonal proteins that may be involved in targeting and localization of Caspr2 include members of the protein 4.1 family and proteins containing PDZ domains. Caspr2 and Nrx-IV share in their cytoplasmic tail a high sequence homology with the erythrocyte transmembrane protein glycophorin C. This protein is found in a ternary complex also containing protein 4.1 and the membrane-associated guanylate kinase protein p55 (Marfatia et al., 1997). The cytoplasmic domain of Caspr has been shown to interact with protein 4.1 from rat brain (Menegoz et al., 1997), and Nrx-IV associates directly with the N-terminal region of Coracle, a *Drosophila* protein 4.1 homolog (Ward et al., 1998). Genetic analyses in *Drosophila* indicate interdependence between protein 4.1/Coracle and Nrx-IV for correct localization. In Nrx-IV mutants, protein 4.1/Coracle is mislocalized and is not found in septate junctions (Baumgartner et al., 1996). In *coracle* null flies, Nrx-IV reaches the lateral membrane but is not subsequently maintained at the septate junctions (Ward et al., 1998). Another protein that is required for the proper localization of Nrx-IV is Disc Lost (Bhat et al., 1999). This protein contains four PDZ domains, two of which interact with the C-terminal tail of Nrx-IV (Bhat et al., 1999). In analogy with Nrx-IV and glycophorin C, it is likely that the localization of Caspr2 to the juxtaparanodal region may also involve a complex interaction with cytoplasmic proteins, such as members of the protein 4.1 family and PDZ domain–containing proteins.

Possible Function of Caspr Family Members in Ion Channel Clustering

The remarkable differentiation of the myelinated axon into distinct domains is thought to be regulated by interactions with glial cells (i.e., glial contact and soluble factors), as well as by cytoskeleton-associated cytoplasmic proteins within the axon (Dugandzija-Novakovic et al., 1995; Vabnick et al., 1996; Kaplan et al., 1997; Waxman, 1997; Rasband et al., 1998, 1999). For example, during the first postnatal week in the rat PNS, Na^+ channels cluster initially at sites adjacent to the edges of processes extended by newly myelinating Schwann cells. Longitudinal growth of these processes causes displacement of the clusters until ultimately two neighboring clusters appear to fuse, thus forming a new node of Ranvier (Vabnick et al., 1996). These results indicate that axoglial interaction induces the clustering of Na^+ channels at the node of Ranvier. Evidence for similar requirements for axon–glia interactions is emerging for the clustering of K^+ channels (Wang et al., 1995; Rasband et al., 1998; Vabnick et al., 1999). During development of the rat optic nerve, Caspr2 is found at initial sites of K^+ channel clustering, suggesting that it may play a role in the localization of these channels. In addition, ultrastructural analysis using immunoelectron microscopy revealed granular staining of Caspr2 in the axonal cytoplasm. Similar staining has been reported for K^+ channels at the juxtaparanodes (Wang et al., 1993), as well as for Na^+ channels in the nodes of Ranvier (Joe and Angelides, 1992). In these cases, the close proximity of these cytoplasmic granules to the final location of ion channels on the membrane prompt the speculation that they reflect part of the axonal machinery involved in the targeting of ion channels (Wang et al., 1993). Furthermore, the presence of both Caspr2 and K^+ channels in these cytoplasmic structures may raise the possibility that they are found in a complex of membrane proteins that is assembled before reaching the membrane. Finally, it has recently been shown that axotactin, a secreted member of the neurexin superfamily, is a glial signal that regulates the electrical properties of axonal membrane in *Drosophila* (Yuan and Ganetzky, 1999). It was proposed that this molecule might be required for the expression, localization, or clustering of ion channels. The association of Caspr2 with K^+ channels at the neuronal membrane may indicate a role for members of this growing family of proteins in the modulation of ion channel clustering that is required for proper conduction of the nerve impulse.

Coupling of Caspr2 to K^+ Channels Required an Additional Component

Is the interaction between Caspr2 and K^+ channels direct or mediated through an additional protein? Initial experiments designed to address this question using expression of Caspr2, Kv1.2, and Kvβ2 in a heterologous system (data not shown) indicate that an additional component is probably required. The C-terminal tail of both Caspr2 and Kv1.2 contains a consensus sequence for the PDZ domain. This is particularly intriguing, since PDZ-containing proteins have been implicated in the clustering of ion channels and other membrane proteins within distinct membrane domains (Sheng, 1997). We have shown that the C-terminal peptide of Caspr2, but not a peptide that is missing the last hydrophobic amino acid, is sufficient to pull down Kv1.2 from brain membrane lysate. Furthermore, a peptide derived from Kv1.2 that includes its PDZ binding site pulled down Caspr2 from rat brain. Deletion of the last valine, a residue known to be essential for Kv1.2 interaction with PDZ domains, from the peptide completely abolished its ability to pull down Caspr2. Thus, it is likely that the observed association between Caspr2 and Kv1.2 reflects their interaction with a common protein containing PDZ domains. Such an association would resemble the interaction between the Shaker K^+ channel and the cell recognition molecule Fasciclin II in *Drosophila*, which interacts through the PDZ-containing protein Discs-large (Zito et al., 1997). A variation on this theme exists at the node of Ranvier, where the association of Na^+ channels and the cell adhesion molecules Neurofascin and NrCAM, through the cytoplasmic anchor Ankyrin-G, has been suggested as a mechanism for the initial localization and clustering of these channels (Lambert et al., 1997).

In summary, we have described the identification of Caspr2, a novel member of the NCP family that is localized to the juxtaparanodal regions in myelinated nerves. In analogy with the proposed role of Caspr in mediating axon–glia interactions (Einheber et al., 1997; Menegoz et al., 1997), Caspr2 might interact with a ligand present on the adaxonal glial membrane in the juxtaparanodal region. However, in contrast to Caspr, the presence of a PDZ domain–binding motif in the cytoplasmic region of Caspr2 may enable it to stabilize the localization of K^+ channels in the juxtaparanodal region. This could be one mechanism by which glial cells can control the localization of various proteins, including ion channels, to specific domains in the axonal membrane.

Experimental Procedures

Cloning of Mouse and Human *caspr2* cDNA

EST R13972 was used to clone the full-length human cDNA from brain and spinal cord libraries, as previously described (Peles et al., 1997). The mouse cDNA was isolated from fetal brain library using a fragment (800 bp HindIII fragment from the 5′ end) of the human *caspr2* cDNA. DNA sequence determination was carried out using the dideoxy-chain termination method with Sequenase 2.0 (United States Biochemical, Cleveland, OH). Sequencing was performed on both strands by priming with synthetic oligonucleotides. An EcoRI fragment containing full-length human *caspr2* was subcloned into pCDNA3 (Invitrogen) to generate pC-hCSP2. Construction of Caspr and contactin expression vectors was previously described (Peles et al., 1995, 1997). To generate a glutathione S-transferase (GST) fusion protein containing the cytoplasmic tail of human Caspr2 (GST–Caspr2CT), the corresponding region (amino acids 1284–1331) was amplified by PCR and cloned into pGEX-4T (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Northern Blot Analysis

DNA fragments (2.1 or 2.3) corresponding to positions 286–2725 or 2148–4448 of the human *caspr2* or *caspr* genes, respectively, were isolated, labeled by random priming, and used as probes. Hybridization to multiple tissue Northern blots (MTN Blots, Clontech, Palo Alto, CA) was carried out as previously described (Peles et al., 1997).

Generation of Antibodies

Polyclonal antibodies against Caspr2 were generated by immunizing rabbits with a GST fusion protein composed of the entire cytoplasmic domain of human Caspr2 (antibody 4344). Antibodies against GST were removed by passing the antiserum through a column of Sepharose–GST. Affinity purification was then performed by loading the unbound material on a column of Sepharose–GST–Caspr2CT. Bound antibodies were eluted with 100 mM Na^+ citrate (pH 2.8), and eluted material was dialyzed against phosphate-buffered saline (PBS). The generation and characterization of mouse monoclonal antibodies to K^+ channel (Bekele-Arcuri et al., 1996) and Na^+ channel (Rasband et al., 1999) polypeptides have been previously described.

For generation of monoclonal antibodies against Caspr, mice were immunized with a fusion protein in which the extracellular domain of Caspr was fused to the Fc region of human immunoglobulin G (IgG; Ig fusion). This recombinant protein was expressed in HEK293 cells and purified from the culture supernatant using protein A chromatography (Peles et al., 1995). After four injections at 3 week intervals, spleens were fused to NSO myeloma cells. Hybridoma supernatants were screened by immunoprecipitation of Caspr from rat NB neuroblastoma lysates. Subcloning of positive hybridomas was done using standard techniques.

Immunoprecipitation and Immunoblot Analysis

Preparation of rat brain membranes, immunoprecipitation from solubilized membrane preparations, and immunoblotting were performed as previously described (Peles et al., 1995, 1997). For peptide pull-down experiments, 30 μg of N-terminally biotinylated peptides derived from the C-terminal tail of Caspr2 (positions 1317–1331), Caspr (positions 1317–1331 of the human protein), Caspr2 C-terminal peptide lacking the last isoleucine (Caspr2-I), kv1.2 (positions 488–489 of the rat protein), or a Kv1.2 peptide lacking the last valine (Kv1.2-V) were bound to avidin-agarose (Pierce, Rockford, IL). Precipitation from rat brain lysate was carried out as described above for immunoprecipitation.

Immunohistochemistry and Immunofluorescence

For immunohistochemical staining of tissue sections, rats were perfused through the transcardiac route with 4% paraformaldehyde (PFA) in PBS. Brains were dissected and incubated for several days in a solution of 1% PFA and 20% sucrose. Staining was done using 35–50 μm floating sections as described below, with the exception that detection of bound antibodies was accomplished using the avidin–biotin complex (ABC) procedure (Vector Laboratories, Burlingame, CA) followed by diaminobenzidine (DAB) and hydrogen peroxidase reaction.

For double- and triple-labeling experiments, rat optic nerves were dissected and fixed in 4% paraformaldehyde in 0.1 M PB (pH 7.2) and then transferred to 20% sucrose (w/v) in 0.1 M PB for 3 hr. The nerves were then frozen in OCT mounting medium (Tissue-Tek), and 10 μm sections were cut on a cryostat. Alternatively, nerves were directly frozen in OCT after dissection. The preparation was then permeabilized for 1 hr with 0.1 M PB (pH 7.4), containing 10% goat serum and 0.3% Triton X-100 (PBTGS). Secondary antibodies were obtained from Accurate Chemical (Westbury, NY) or Jackson Immunoresearch (West Grove, PA). In some experiments, biotinylated secondary antibody was used, followed by streptavidin-conjugated FITC. The coverslips were incubated with Caspr2 rabbit polyclonal antibody (1:100) overnight. The coverslips were then washed, and anti-rabbit FITC H+L (JGZ-097003) (1:50) was added for 2 hr. The

coverslips were washed again, and a mouse monoclonal Na^+ channel antibody (1:10,000) was added overnight. The coverslips were washed, and anti-mouse aminomethylcoumarin acetate antibody (JGM-155003) (1:50) was added for 1 hr. After washing, rabbit polyclonal Caspr antibody (1:2,500) was added for 30 min. The coverslips were washed again, and anti-rabbit Cy3 antibody (JGZ-167003) (1:2,000) was added for 30 min. The coverslips were then washed once in PBTGS, once in 0.1 M PB, and once in 0.05 M PB and then dried and mounted for observation. Immunofluorescence slides were viewed and analyzed using a Deltavision wide-field deconvolution microscope (Applied Precision), a BioRad confocal microscope (BioRad, Richmond, CA), or a Nikon Microphot equipped with a Hamamatsu cooled charge-coupled device camera.

Electron Microscopy

Coronal vibratome sections of the cerebellum (40 μm thick) were prepared from acrolein-perfused adult male Sprague-Dawley rats (Einheber et al., 1996). Prior to immunostaining, the sections were subjected to the "freeze-thaw" technique (Milner et al., 1998) to improve antibody penetration. For immunolabeling, free-floating permeabilized sections were incubated with affinity-purified Caspr2 polyclonal antibody (1:250) for 1 day at room temperature followed by 1 day at 4°C. Bound antibody was detected by the ABC method with the chromagen DAB. Immunolabeled sections were postfixed for 1 hr in 2% osmium tetroxide and flat embedded in EMbed as described previously (Einheber et al., 1996). Regions of the arbor vitae in the cerebellum, which contain Caspr2-immunolabeled myelinated fibers, were selected and glued onto EMbed-cured blocks. Ultrathin sections (50 nm thick) were cut from the plastic–tissue interface, counterstained with uranyl acetate and lead citrate, and examined with a Philips CM10 electron microscope.

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GenBank Accession Number

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