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Caspr3 and Caspr4, Two Novel Members of the Caspr Family Are Expressed in the Nervous System and Interact with PDZ Domains

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The NCP family of cell-recognition molecules represents a distinct subgroup of the neurexins that includes Caspr and Caspr2, as well as Drosophila Neurexin-IV and axotactin. Here, we report the identification of Caspr3 and Caspr4, two new NCPs expressed in nervous system. Caspr3 was detected along axons in the corpus callosum, spinal cord, basket cells in the cerebellum and in peripheral nerves, as well as in oligodendrocytes. In contrast, expression of Caspr4 was more restricted to specific neuronal subpopulations in the olfactory bulb, hippocampus, deep cerebellar nuclei, and the substantia nigra. Similar to the neurexins, the cytoplasmic tails of Caspr3 and Caspr4 interacted differentially with PDZ domain-containing proteins of the CASK/Lin2-Veli/Lin7-Mint1/Lin10 complex. The structural organization and distinct cellular distribution of Caspr3 and Caspr4 suggest a potential role of these proteins in cell recognition within the nervous system.

INTRODUCTION

Cell adhesion and recognition molecules mediate an astonishing complexity of cellular interactions required for the establishment of the correct pattern of connections in the developing nervous system. These molecules are divided by their structural characteristics into several protein families including members of the immunoglobulin superfamily (Brummendorf and Lemmon, 2001), cadherins (Redies, 2000; Shapiro and Colman, 1999), protocadherins (Suzuki, 2000), proteoglycans (Bandtlow and Zimmermann, 2000), and the neurexins (Missler and Sudhof, 1998). The neurexin superfamily consists of a growing list of both secreted and transmembrane proteins (Missler and Sudhof, 1998). Six different neurexins (i.e., Iα–IIIα and Iβ–IIIβ), generated from three distinct genes (Ushkaryov *et al.*, 1992; Ushkaryov and Sudhof, 1993), undergo extensive alternative splicing to generate a large number of isoforms that are differentially expressed throughout the brain (Ullrich *et al.*, 1995; Puschel and Betz, 1995). The extracellular region of the neurexins binds to several membrane bound ligands, including neuroligins (Ichtchenko *et al.*, 1995; Nguyen and Sudhof, 1997; Scheiffele *et al.*, 2000) and dystroglycan (Sugita *et al.*, 2001), suggesting their involvement in cell–cell interactions and synapse formation.

The NCP family (for Neurexin-IV / Caspr / Paranodin; Bellen *et al.*, 1998) represents a distinct subgroup of the neurexins that includes mammalian Caspr (Contactin associated protein, also known as Paranodin, Peles *et al.*, 1997; Menegoz *et al.*, 1997) and Caspr2 (Poliak *et al.*, 1999), Drosophila Neurexin-IV (Baumgartner *et al.*, 1996) and axotactin (Yuan and Ganetzky, 1999), as well as Intexin and Nlp in *C. elegans* (L. Haklai-Topper and E. Peles, unpublished observations). Both the neurexins and members of the NCP subgroup mediate cell–cell interactions. However, while the neurexins are found in synapses and are thought to mediate the interactions between neurons (Song *et al.*, 1999; Ushkaryov *et al.*, 1992), NCP proteins mediate neuron–glial interactions and in invertebrates, glial–glial contact (Baumgartner *et al.*, 1996; Yuan and Ganetzky, 1999; Bhat *et al.*, 2001). In myelinated axons, Caspr is concentrated with contactin at the paranodal junctions that are formed between axons and the terminal loops of oligodendrocytes and myelinating Schwann cells (Einheber *et al.*, 1997; Menegoz *et al.*, 1997; Rios *et al.*, 2000). Caspr is essential for the generation of these junctions, and its absence in mice lacking galactolipids, contactin, or Caspr itself, results in paranodal abnormalities, mislocalization of K^+ channels and decrease in nerve conduction (Bhat *et*

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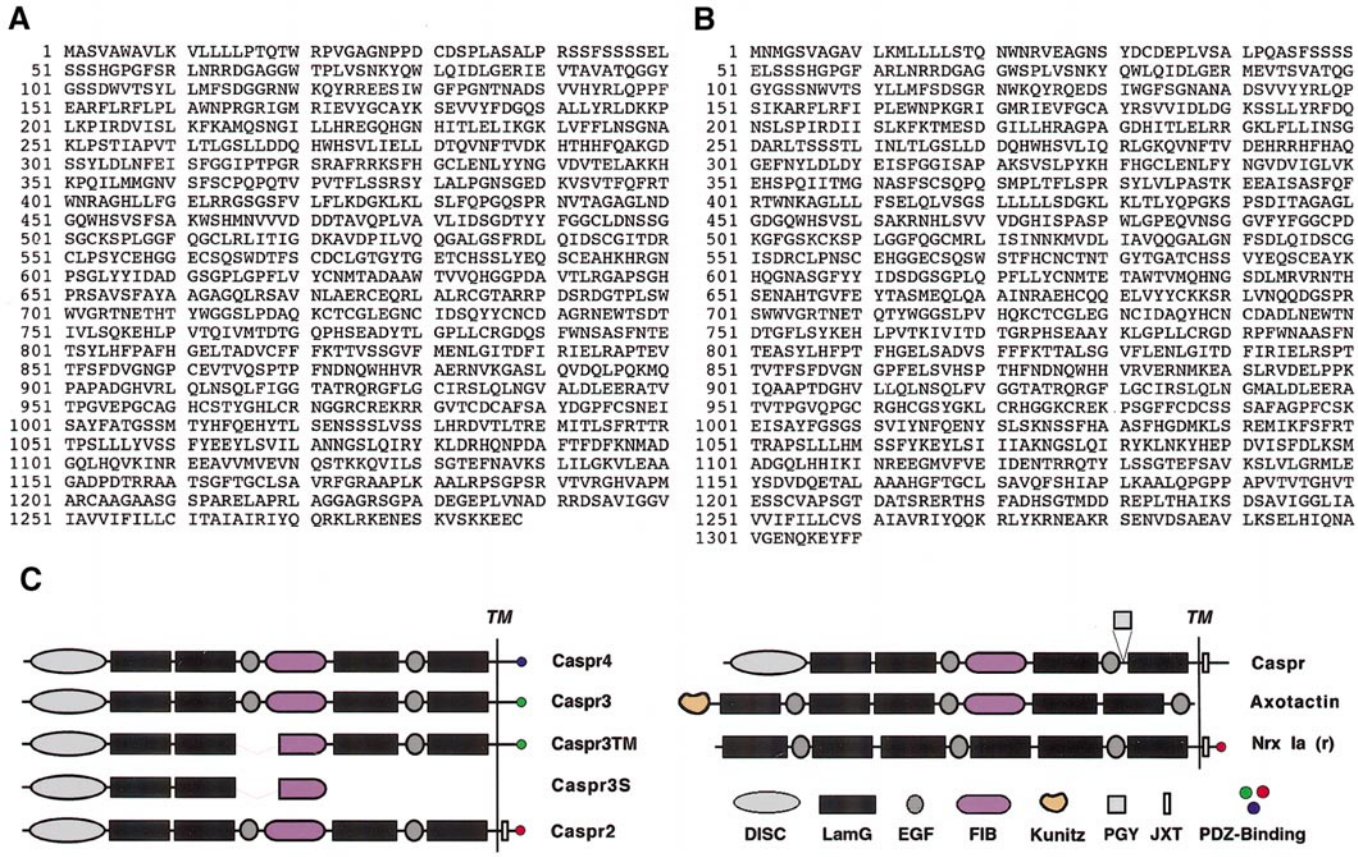


FIG. 1. Amino acid sequences and domain organization of Caspr3 and Caspr4. Amino acids sequence of human Caspr3 (A) and mouse Caspr4 (B). (C) Schematic organization of the Caspr proteins. All Caspr proteins contain a discoidin-like domain (DISC), a region similar to fibrinogen (FIB), four laminin G domain (LamG; also known as LNS domains), and two EGF repeats. A repeat region of proline glycine and tyrosine residues (PGY) is found only in Caspr and not in Caspr2–Caspr4. The discoidin and the fibrinogen-like domains are not found in the related mammalian neurexins. For comparison, the domain organization of rat neurexin Iα (Nrx Ia) and *Drosophila* axotactin is also shown. Similar to Caspr2 and the neurexins, Caspr3 and Caspr4 each have a short cytoplasmic domain that contains a carboxy-terminal binding site for PDZ domains. However, the cytoplasmic juxtamembrane region (JXT) of Caspr3 and Caspr4 does not contain the conserved protein 4.1 binding site found in Caspr, Caspr2, and the neurexins (see also Fig. 7A). Alternative splicing generates two additional forms of Caspr3; Caspr3TM and Caspr3S, both missing exons 8–10, resulting in proteins that lack the first EGF domain and a small part of the fibrinogen-like region. Caspr3S lacks also exons 13–24 and may represent a secreted isoforms of Caspr3. TM, transmembrane domain.

al., 2001; Boyle *et al.*, 2001; Dupree *et al.*, 1999; Poliak *et al.*, 2001). In contrast to Caspr, Caspr2 is found at the juxtaparanodal region where it is physically associated with the K^+ channel α-subunits, Kv1.1 and Kv1.2 and their cytoplasmic Kvβ2 subunit (Poliak *et al.*, 1999). Another member of the NCPs is Drosophila axotactin, a secreted molecule produced by glial cells and affecting nerve conduction by a yet unknown mechanism (Yuan and Ganetzky, 1999). The presence of axotactin in the fly suggested the existence of additional members of the neurexin-superfamily in vertebrates. In the present study, we describe the identification and characterization of Caspr3 and Caspr4, two novel and closely related members of the NCP group, which are uniquely expressed in the nervous system.

RESULTS

Caspr3 and Caspr4 Are Two New Members of the NCP Family

The presence of two different human Caspr genes (Peles *et al.*, 1997; Poliak *et al.*, 1999) and an additional secreted member of this family in *Drosophila* (Yuan and Ganetzky, 1999), has prompted us to search for additional Caspr and Caspr2 homologues in the vertebrate database. One EST was found (GenBank Accession No.: AI148152) and used as a specific probe to clone human Caspr3 cDNA from an astrocytoma-derived Hs683 cell line cDNA library. A fragment of this cDNA was further used to clone mouse Caspr4 from a fetal brain

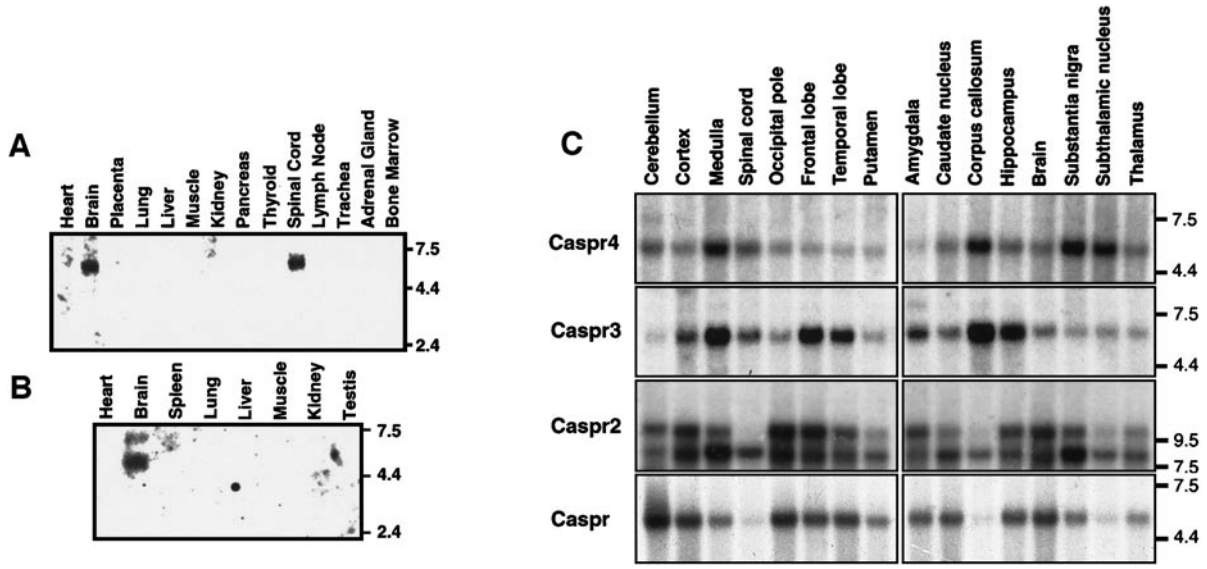
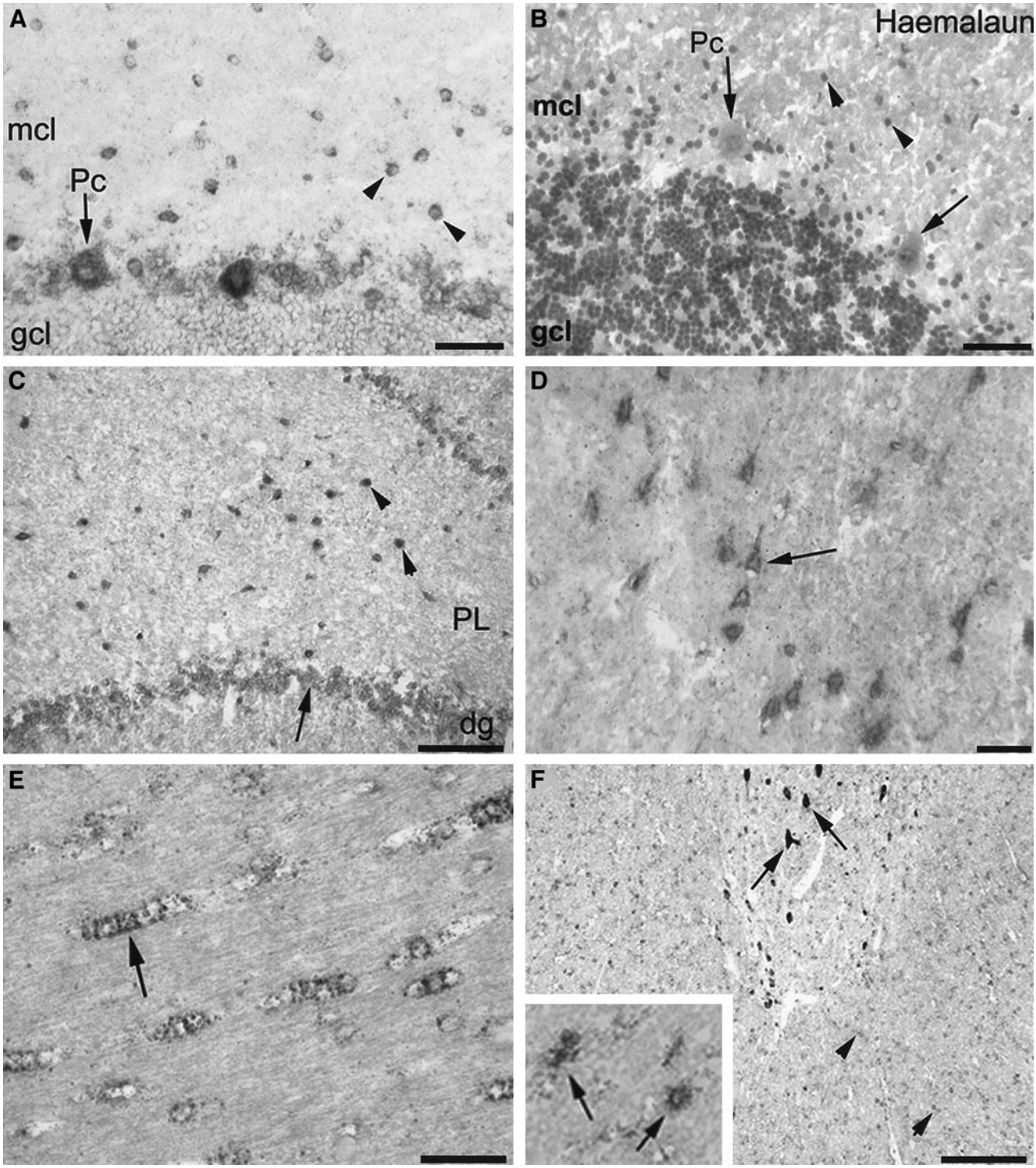


FIG. 2. Expression of Caspr3 and Caspr4 mRNA is restricted to the nervous system. Northern blots containing mRNA from various human (A) or mouse (B) tissues were hybridized with a specific probe for human Caspr3 (A) or mouse Caspr4 (B). The autoradiograms are shown along with molecular weight markers in Kb. (C) Expression of the four Caspr genes in different regions of the human nervous system.

cDNA library (detailed in materials and methods). A sequence containing the entire human Caspr4 cDNA was compiled from various ESTs according to the mouse cDNA. Partial sequences corresponding to human Caspr3 and Caspr4 cDNAs (KIAA1763 and KIAA1714, respectively) can also be found in the HUGE database of the Kasuza DNA Research Institute (Nagase *et al.*, 2000). Human Caspr3 and mouse Caspr4 cDNAs have open reading frames that encode for 1288 and 1310 amino acids, respectively (Figs. 1A and 1B). An alignment of the amino acid sequence of all Caspr proteins has revealed that Caspr3 and Caspr4 exhibit 70% identity and are thus more related to each other than to Caspr, Caspr2 or to human EST sequence (AK056528), which most likely represents Caspr5 (Table 1). The structural organization of the encoded Caspr3 and Caspr4 polypeptides is very similar to that of Caspr and Caspr2, all having the hallmarks of type I transmembrane proteins and sharing an overall structural similarity with the neurexins (Missler and Sudhof, 1998). Both Caspr3 and Caspr4 consist of a large extracellular domain, a single membrane-spanning domain and a short cytoplasmic region at their carboxy-terminus. Like the other family members, their extracellular region is composed of several defined domains, including discoidin and fibrinogen-like domains, two EGF repeats and four laminin G domains (also known as LNS domains; Fig. 1C). The latter comprise a globular, lectin-like fold found in many proteins including the G domain of laminin A, agrin, slit and perlecan (Rudenko

FIG. 3. Expression of Caspr3 in the adult human nervous system. *In situ* hybridization of Caspr3 was performed on tissue sections from cerebellum (A), hippocampus (C, D), corpus callosum (E), and spinal cord (F). (A) In the cerebellum, Caspr3 expression was detected in Basket cells in the molecular cell layer (arrowhead) and in Purkinje cells (arrow). (B) Haemalaun cellular staining of an adjacent tissue section for comparison. (C and D) In the hippocampus, Caspr3 expression was detected in the granule cells of the dentate gyrus (C, arrow), in neurons of the polymorphic layer (C, arrowheads) and in pyramidal neurons of CA1 (D, arrow). (E) Sagittal section of the corpus callosum showing expression of Caspr3 in oligodendrocytes (E, arrow). Note the chain-like arrangement of the cell bodies typical for oligodendrocytes aligning along myelinated axons. (F) Transverse section of the spinal cord showing high expression of Caspr3 in motor neurons in the ventral horn (arrows). In addition, Caspr3 expression was detected in small cells throughout white and gray matter. Caspr3 transcripts could also be detected within the thin processes of these oligodendroglial cells (F, inset, arrows). *In situ* hybridization with the sense probe for Caspr3 did not show any hybridization signal (not shown). Abbreviations: dg, dentate gyrus; gel, granule cell layer; gm, gray matter; mcl, molecular cell layer; NSE, neuron specific enolase; Pc, Purkinje cell; PL, polymorphic layer; PLP, proteolipid protein; wm, white matter. Scale bar: (A, B) 30 μm; (C) 200 μm; (D–F) 50 μm; (G–K) 200 μm.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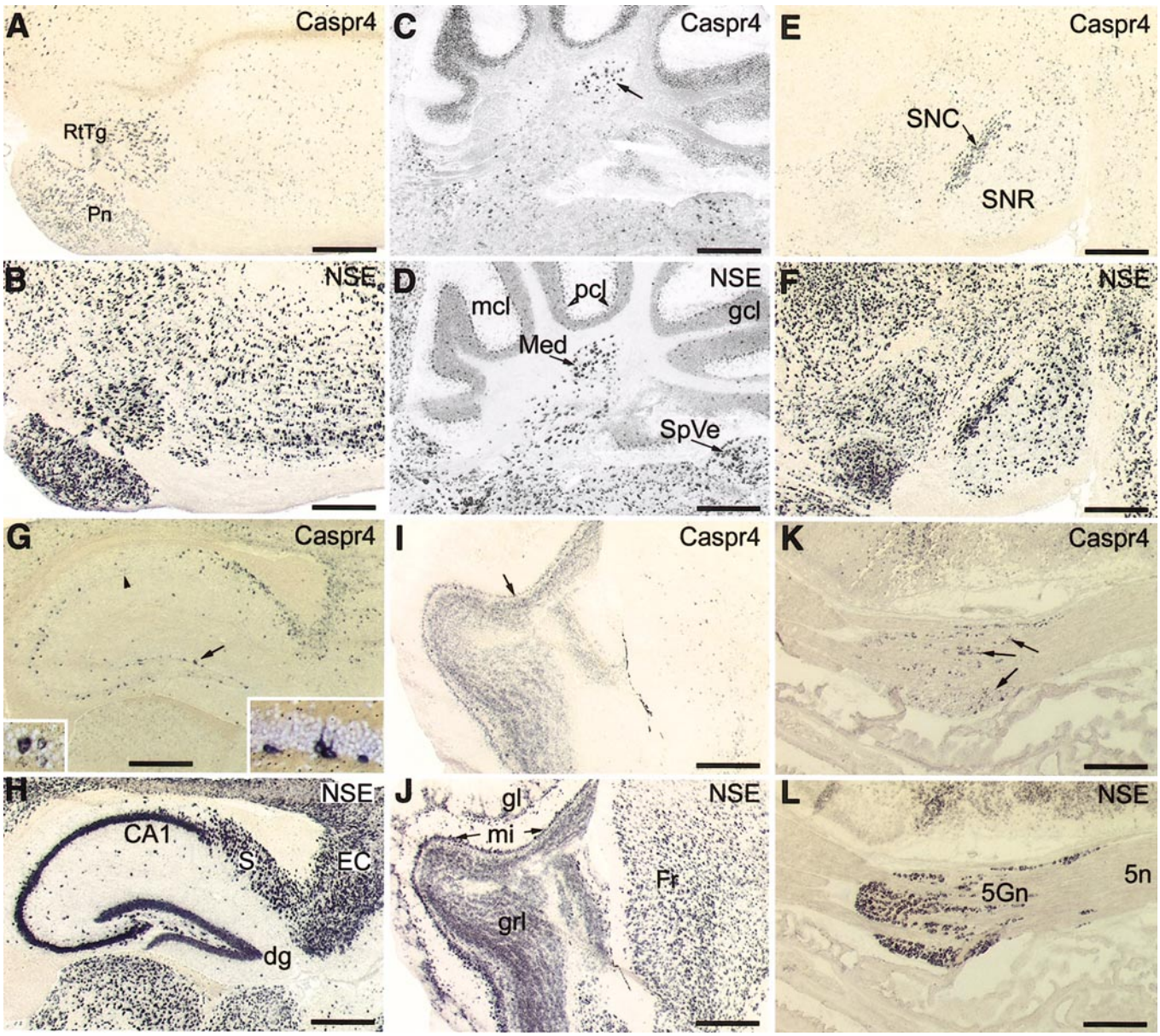

FIG. 4. Expression of Caspr4 mRNA in mouse CNS. *In situ* hybridization for Caspr4 was performed on sagittal sections of mouse brain (A, C, E, G, I, K). For each sample, the expression pattern of NSE is shown on an adjacent tissue section for comparison (B, D, F, H, J, L). (A) In the brain stem, prominent expression was detected in the pontine and reticulotegmental pontine nuclei. (C) In the cerebellum, high expression levels of Caspr4 could be localized in the deep cerebellar nuclei (arrow), while lower levels were detected in the granule cells. (E) Expression of Caspr4 in neurons of the compact area of the substantia nigra (arrow). Lower levels of Caspr4 are found also in neurons in the reticular area of substantia nigra (SNR). (G) In the hippocampus, Caspr4 expression was predominant in the basket cells of the dentate gyrus (arrow, right inset), in basket cells within the Pyramidal cell layers CA1-CA3 (arrowhead, left inset) and in the superficial layer of the subiculum. In the entorhinal cortex, Caspr4 expressing cells were found mostly in layer VI and III (G). (I) In the olfactory bulb, expression of Caspr4 was seen in the granule cells and in mitral cells (arrow). Comparison with NSE (J) shows that neurons of the glomeruli were negative. (L) Expression of Caspr4 in peripheral ganglion neurons such as the trigeminal ganglion. Myelinating Schwann cells of the trigeminal nerve were negative. Abbreviations: dg, dentate gyrus; gcl, granule cell layer of the cerebellum; gl, glomeruli; grl, granule cell layer of the olfactory bulb; EC, endorhinal cerebral cortex; Fr, frontal cerebral cortex; 5Gn, Trigeminal ganglion; mcl, molecular cell layer; Med, medial cerebellar nuclei; mi, Mitral cell layer; 5n, trigeminal nerve; NSE, neuron specific enolase; pcl, Purkinje cell layer; Pn, pontine nuclei; RtTg, reticulotegmental nucleus of the pons; S, subiculum; SNC, compact area of substantia nigra; SNR, reticular area of substantia nigra; SpVe, spinal vestibular nuclei. Scale bar: (A–J) 500 μm; (K, L) 200 μm.

TABLE 1
Homology among the Caspr Proteins

	Caspr	Caspr2	Caspr3	Caspr4	Caspr5	Chromosome
Caspr	—	43%	37%	38%	38%	17q21
Caspr2		—	47%	48%	49%	7q35
Caspr3			—	70%	53%	9q11
Caspr4				—	58%	16q22
Caspr5					—	2q14

Note. Numbers indicate the percentage of identical amino acids calculated by Blast-alignment. The chromosomal location of the different genes in human is shown on the left. GenBank Accession Nos: human Caspr, NM_003632; human Caspr2, NM_014141; human Caspr3, AF333769; human Caspr4, AF463518; human Caspr5, AK056528.

et al., 1999, 2001), and is thought to mediate cell-matrix interactions. As depicted in Fig. 1C, we have also cloned two alternatively spliced forms of Caspr3 termed hCaspr3TM and hCaspr3S, which contain open reading frames of 1154 and 612 amino acids, respectively, and lack the first EGF domain and a small part of the fibrinogen-like region. In addition, Caspr3S ends after the fibrinogen-like region and may represent a secreted isoform of Caspr3. Caspr3 and Caspr4 contain a short cytoplasmic domain of 21 and 44 amino acids, respectively. Both display a very similar sequence at their juxtamembrane region but lack the protein 4.1-binding sequence found in Caspr and Caspr2 (see Fig. 7A for sequence alignment). Similar to Caspr2 and all the neurexins (but not Caspr), the C-termini of Caspr3 and Caspr4 contain a binding site for PDZ domains (Bhat *et al.*, 1999; Butz *et al.*, 1998; Hata *et al.*, 1996; Missler and Sudhof, 1998). Thus, Caspr3 and Caspr4 are two novel members of the NCP family and are structurally similar to the known Caspr and Caspr2 proteins.

Caspr3 and Caspr4 Are Predominantly Expressed in the Nervous System

Analysis of the expression of Caspr3 mRNA in various human tissues by Northern blots revealed the presence of a single 6.1 kb transcript in brain and spinal cord (Fig. 2A). A similar analysis performed using a specific probe to mouse (Fig. 2B) or human (Fig. 2C) Caspr4, detected a transcript of 5.1 kb in brain. In addition, a minor 6.3-kb transcript was detected in mouse but not in human brain tissues. Similar to Caspr3, Caspr4 expression was not detected in any of the other tissues examined. However, it is worth noting that several ESTs corresponding to Caspr3 and Caspr4 isolated from tissues outside the nervous system could be found in the databases (UniGene Clusters: Hs.212839 and Hs.156829, respectively), suggesting that these genes may be expressed at lower levels in other tissues as well. We next compared the expression of Caspr3 and Caspr4 in different regions of the adult human CNS to that of the other members of the Caspr family. As depicted in Fig. 2C, although both Caspr3 and Caspr4 transcripts could be detected in all the regions examined, they exhibited a distinct expression pattern. With the exception of the corpus callosum and the medulla, where an intense signal for both genes was observed, the highest expression of Caspr3 was detected in the temporal and frontal lobes and the hippocampus, while Caspr4 was vastly found in the subthalamic nucleus and the substantia nigra. The distribution of Caspr3 and Caspr4 mRNA was clearly different from that observed for Caspr and Caspr2, which displayed an overlapping expression pattern (Fig. 2C and Poliak *et al.*, 1999). These results demonstrate that both Caspr3 and Caspr4 are primarily expressed in the nervous system where they exhibit a distinct pattern of expression from that of other members of this family, suggesting that they may have different functions in the CNS.

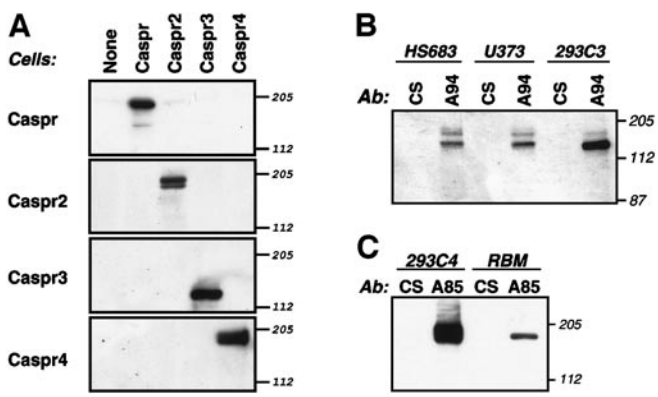

FIG. 5. Specificity of the antibodies to different Casprs. (A) HEK-293 cells were transfected with the different Caspr genes as indicated on top (Cells). Untransfected cells (None) were used as a negative control. Cell lysates were subjected to immunoprecipitation and Western blot analysis using antibodies to the different Caspr proteins as indicated on the left of each panel. Molecular weight markers in kDa are shown on the right. (B) Expression of the endogenous Caspr3 protein. Lysates from HS683 and U373MG glial-derived cell lines, or HEK-293 cells transfected with Caspr3TM cDNA (293C3) were subjected to immunoprecipitation with specific antibodies to Caspr3 (A94), or with the preimmune serum (CS) as indicated, followed by immunobloting with anti-Caspr3 antibody. (C) Solubilized rat brain membranes (RBM) or HEK-293 cells transfected with Caspr4 (293C4) were subjected to immunoprecipitation with specific antibodies to Caspr4 (A85), or with the preimmune serum (CS). The immune-complexes were separated on SDS gels and blotted with an antibody to Caspr4.

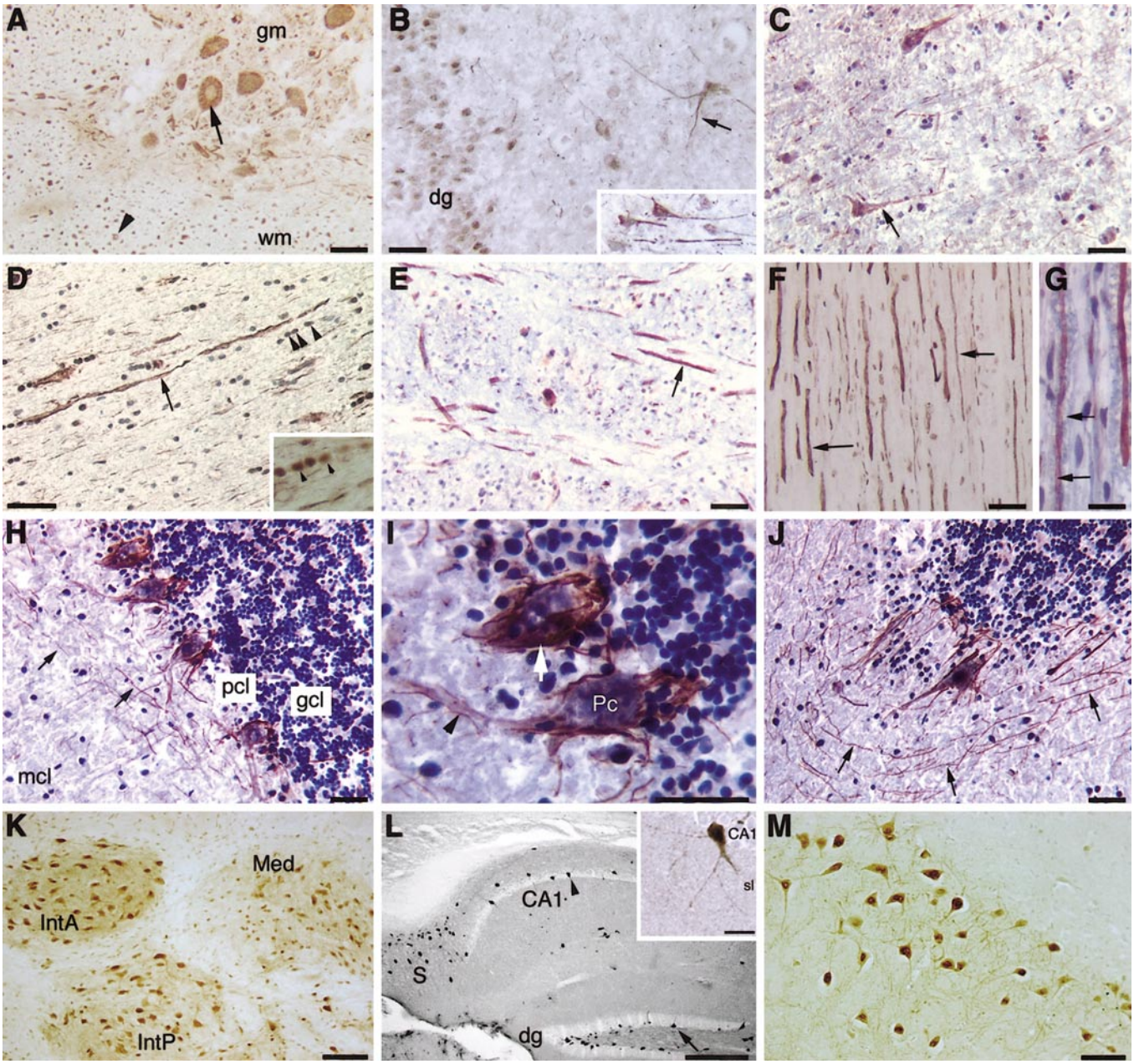

FIG. 6. Immunolocalization of Caspr3 and Caspr4 in human and mouse nervous systems. Immunoperoxidase staining was performed on sections of human (A–J) or mouse (K–M) tissues using Caspr3 (A–J) or Caspr4 (K–M) antibodies. (A) Motor neurons in the spinal cord show intense Caspr3 immunoreactivity (arrow; gm, gray matter). In the white matter (wm), staining is associated with ascending and descending axons (arrowhead). (B) In the hippocampus, granule cells of the dentate gyrus (dg), large pyramidal-shaped neurons within the polymorphic cell layer of the dentate gyrus (arrow), and pyramidal cells (inset) express Caspr3. (C) In the cerebral cortex, cell bodies and dendrites of pyramidal cells of the third and fifth layers showed Caspr3 immunoreactivity (arrow). In addition, small round cells throughout all layers were positive. Prominent expression of Caspr3 protein was detected along axons (arrows) in the corpus callosum (D), medulla (E), and in femoral nerves (F and G). In addition, a clear staining of Caspr3 was observed in oligodendrocyte cell bodies aligning along the longitudinal axonal axis of the tracts in the corpus callosum (arrowheads and inset in D). (H, J) Sections of the cerebellum, showing intense labeling of Caspr3 along basket cell axons projecting parallel to the Purkinje cell layer (pcl; arrows). (I) Higher magnification showing the expression of Caspr3 in the axons of the inhibitory basket cells that terminates at cell bodies of Purkinje cells (Pc, arrow). In addition, weak Caspr3 immunoreactivity could also be seen in the Purkinje cell themselves and their dendritic shaft (arrowhead), as well as occasionally in their axons located in the granular cell layer (gcl). (K) Caspr4 immunoreactivity is detected in cell bodies and dendrites of cells in the cerebellar deep nuclei (IntA, IntP, Med). (L) In the hippocampus, Caspr4 is found in basket cells of the dentate gyrus (dg; arrow) and within the pyramidal cell layer (CA1; arrowhead and inset), and in cell bodies and dendrites of cells in the superficial layer of the subiculum (S). (M) Strong immunoreactivity signal of Caspr4 in cell bodies and dendritic shafts of cells in the compact area of substantia nigra. Abbreviations: dg, dentate gyrus; gcl, granule cell layer; gm, grey matter; IntA, interpositus anterior cerebellar nucleus; IntP, interpositus posterior cerebellar nucleus; Med, medial deep cerebellar nucleus; Pc, Purkinje cell; pcl, Purkinje cell layer; S, subiculum; wm, white matter. Scale bars: (A–F, H–J, M) 50 μm; (G) 20 μm; (K) 100 μm; (L) 200 μm.

Distribution of Caspr3 and Caspr4 Transcripts in Human and Mouse Tissues

We next determined the expression of Caspr3 and Caspr4 using *in situ* hybridization. The expression of Caspr3 was analyzed in sections of various adult human brain tissues, including the cerebellum, hippocampus, cerebral cortex, medulla, spinal cord, corpus callosum, and in peripheral femoral nerves (Fig. 3). In the cerebellum, Caspr3 was mainly detected in Purkinje cells (Fig. 3A, arrows) and basket cells in the inner molecular cell layer (Fig. 3A, arrowheads). Lower expression could also be detected in cells lying in the Purkinje cell layer and in stellate cells in the outer molecular cell layer. In the hippocampus, Caspr3 was detected in the pyramidal cells of CA1 (Fig. 3D, arrow), CA2 and CA3 areas, in granule cells of the dentate gyrus (Fig. 3C, arrow) and in neurons of the polymorphic layer of the dentate gyrus (Fig. 3C, arrowheads). A strong signal was also detected in neurons in the subiculum as well as in the pre- and parasubiculum (data not shown). In the corpus callosum, high expression of Caspr3 was detected in oligodendrocytes. Caspr3-expressing cells appeared as a chain-like arrangement (Fig. 3E, arrow), resembling the one obtained with the oligodendrocyte marker proteolipid protein (PLP) (data not shown). Caspr3 showed no overlapping signal with GFAP-labeled astrocytes (data not shown), further indicating that it is expressed by oligodendrocytes. In the spinal cord, strong hybridization signals could be seen in the motor neurons of the ventral horn (Fig. 3F, arrows). Comparison with the expression pattern of neuronal specific enolase (NSE) indicated that most of the neurons within the gray matter express Caspr3. In addition, Caspr3 expression could also be detected in oligodendrocytes and their stellate processes within the white matter (Fig. 3F, arrowhead and inset). Similar to the corpus callosum, these cells expressed PLP (data not shown) and showed a typical chain like arrangement (Fig. 3F, arrowhead). Examination of the cerebral cortex revealed that Caspr3 was also expressed in oligodendrocytes within the cortical white matter (data not shown). Finally, in contrast to the CNS (Fig. 3), no specific signal of Caspr3 was detected in peripheral femoral nerves (data not shown), indicating that it is specifically expressed by oligodendrocytes and not by myelinating Schwann cells, although we can not exclude the possibility that Caspr3 expression in Schwann cells was below detection limit.

We next analyzed the expression of Caspr4 in the adult mouse nervous system. The highest expression was detected in a subpopulation of neurons in the medulla and pons, cerebellum, olfactory bulb and the substantia nigra, while lower levels were observed in the cerebral cortex, hippocampus, hypothalamus, caudate putamen, inferior and superior colliculi, and peripheral ganglion cells (Fig. 4). In the brain stem, high expression of Caspr4 was detected in the pontine and reticulotegmental pontine nuclei (Fig. 4A) and in the principle sensory trigeminal nucleus (not shown). In the cerebellum, Caspr4 hybridization signal was detected in the cerebellar deep nuclei and in the granule cells, while Purkinje cells and cells of the molecular layer were negative (Fig. 4C). In the mesencephalon, high expression of Caspr4 was found in the compact area of the substantia nigra (Fig. 4E). Lower expression could also be seen in the reticular area of the substantia nigra (Fig. 4E) and in a subtype of neurons in the superior and inferior colliculi (not shown). In the telencephalon, Caspr4 expression was detected in a subtype of neurons in the hippocampus, cerebral cortex, caudate putamen and olfactory bulb. In the hippocampus, Caspr4 was mostly expressed by basket cells of the dentate gyrus (Fig. 4G, arrow, insert right) and by basket cells within the pyramidal cell layer CA1-3 (Fig. 4G, arrowhead, inset left). In addition, strong hybridization signal was detected in cells within the superficial layer of the subiculum and in layer VI and III of the endorhinal cortex (Fig. 4G). Depending on the cortical area, Caspr4 expression showed different patterns; in the frontal (Fig. 4I) and pariental cortex Caspr4 expressing cells were found in all layers (except layer I), whereas in the primary motor cortex Caspr4 positive cells were mainly found in layer IV and VI (not shown). In the olfactory bulb, Caspr4 was detected in the granule and mitral cells (Fig. 4I, arrow), whereas neurons of the glomeruli were negative. Finally, expression of Caspr4 was also detected in peripheral ganglion neurons such as the trigeminal ganglion cells (Fig. 4K, arrow). This analysis further demonstrated that Caspr3 and Caspr4 are expressed in different cell types. While both genes could be found in distinct neuronal populations throughout the brain, Caspr3, but not Caspr4, is also expressed in oligodendrocytes.

Immunolocalization of Caspr3 and Caspr4

In order to study the distribution of Caspr3 and Caspr4 in the nervous system we have generated polyclonal antibodies against these proteins. As depicted in Fig. 5, antibodies directed against Caspr3 detected a 135-kDa protein from HEK293 cells transfected with Caspr3TM but not from non-transfected or cells transfected with other Caspr family members. Similarly, an antibody directed to the cytoplasmic domain of Caspr4 specifically detected a protein with the apparent molecular mass of 190 kDa from cells transfected with Caspr4 cDNA (Fig. 5A). These

antibodies were specific to Caspr4 and did not cross react with Caspr, Caspr2, or Caspr3. In addition to transfected cells, anti-Caspr3 and anti-Caspr4 antibodies precipitated the endogenous proteins from human glioblastoma cell lines and rat brain membrane lysates, respectively (Figs. 5B and 5C). In HS683 and U373MG cell lines, anti-Caspr3 antibody recognized two proteins in the apparent MW of 135 and 180 kDa, likely corresponding to Caspr3 and Caspr3TM. Finally, antibodies against Caspr and Caspr2 did not recognize Caspr3 or Caspr4 (Fig. 5A), further demonstrating the specificity of our antibodies towards distinct members of the Caspr family.

Immunohistochemical analysis of Caspr3 and Caspr4 using different human and mouse tissues, respectively, revealed an expression pattern that corresponded well to that obtained by *in situ* hybridization. In the spinal cord, strong Caspr3 immunoreactivity was detected in the motor neurons in the ventral horn (Fig. 6A, arrow) and in most other neurons of the gray matter. In the white matter prominent immunoreactivity was associated with ascending and descending axons (Fig. 6A, arrowhead) but no staining of cell bodies was observed. In the hippocampus, Caspr3 protein was detected in granule cells (Fig. 6B), along axons and dendritic shafts of pyramidal shaped neurons within the polymorphic cell layer that are most probably basket cells (Fig. 6B, arrow), in pyramidal cells of CA1, CA2 and CA3 (Fig. 6B, inset), and in cells within the subiculum (not shown). In the cerebral cortex, pyramidal cells of layer III and V and their dendrites showed immunoreactivity (Fig. 6C, arrow), but also small round cells throughout all layers were positive. In the corpus callosum, Caspr3 immunoreactivity was detected along the axons (Fig. 6D, arrow) and in oligodendrocyte cell bodies (Fig. 6D, arrowheads, inset). Similar strong immunoreactivity along axons was also observed in the medulla (Fig. 6E, arrow) and in femoral nerves (Figs. 6F and 6G, arrows). Staining for neurofilaments showed that Caspr3 immunoreactivity was detected only in part of the axons (not shown). The most prominent and clear axonal staining of Caspr3 was detected in the cerebellum (Figs. 6H–6J). Strong Caspr3 immunoreactivity was detected along basket cell axons projecting parallel to the Purkinje cell layer (Figs. 6H and 6J, arrows). These inhibitory basket cells are found within the lower part of the molecular layer and project their axons to the cell body of the Purkinje cells (Fig. 6J, arrowhead). In addition, low immunoreactivity could also be seen in the Purkinje cells themselves and their dendritic shaft (Fig. 6I, arrow). In contrast to the axonal localization of Caspr3, Caspr4 immunoreactivity was mainly detected in cell bodies and dendrites (Fig. 6K–6M). It was detected in limited neuronal populations in various brain tissues, including cells in the cerebellar deep nuclei (Fig. 6K), basket cells of the dentate gyrus (Fig. 6L, arrow), within the pyramidal cell layer of the hippocampus (Fig. 6L, arrowhead, inset), and in the superficial layer of the subiculum (Fig. 6L). The strongest immunoreactivity signal of Caspr4 was obtained in the mesencephalon where it was localized in cell bodies and dendritic shafts of cells in the compact area of the substantia nigra (Fig. 6M). Thus, Caspr3 and Caspr4 exhibit distinct subcellular localization: while Caspr3 is found along axons and in oligodendrocytes, Caspr4 is found in the cell bodies and dendrites of distinct neuronal populations within the brain.

The Cytoplasmic Domains of Caspr3 and Caspr4 Interact with PDZ Domains

The neurexins bind to a multimeric complex of adaptor proteins that consist of CASK/Lin2, Velis/MALS/Lin7 and Mint1/X11α/Lin10 (Butz *et al.*, 1998), creating a potential protein scaffolding center for the generation of specialized membrane domains. The binding of neurexins to this complex is mediated by their interactions with distinct PDZ domains found in the adaptor proteins (Biederer and Sudhof, 2000; Butz *et al.*, 1998). Comparison of the amino acid sequences of Caspr3 and Caspr4 revealed that both proteins contain a putative binding site for PDZ domains in their cytoplasmic tail (Fig. 7A). However, while Caspr4, like Caspr2 and the neurexins, exhibits a classical type II PDZ binding sequence (Songyang *et al.*, 1997; Vaccaro *et al.*, 2001), Caspr3 has a potential binding site for type III PDZ domains (Maximov *et al.*, 1999). To study whether different members of the Caspr family bind to distinct types of PDZ domains, we have examined the ability of immobilized peptides corresponding to the cytoplasmic tails of Caspr2, Caspr3, and Caspr4 to precipitate CASK, Mint1, or Veli. As a positive control, we have used the cytoplasmic domain of RPTPβ, a receptor-like tyrosine phosphatase that interacts with various PDZ domain-containing proteins (Kawachi *et al.*, 1999; Adamsky and Peles, unpublished observations). As depicted in Fig. 7B, Caspr2 peptide precipitated CASK but not Mint1, while Caspr3 interacted with Mint1 but not with CASK, and Caspr4 was able to bind both, CASK and Mint1. No association between members of the Caspr family and Veli was detected. As expected, removing the last amino acid from each of the peptides completely abolished their binding (Fig. 7B), further indicating that the PDZ domains present in the adaptor proteins mediate the observed associations. Furthermore, none of the cytoplasmic tails of the different Caspr proteins interacted with PSD95, a three PDZ

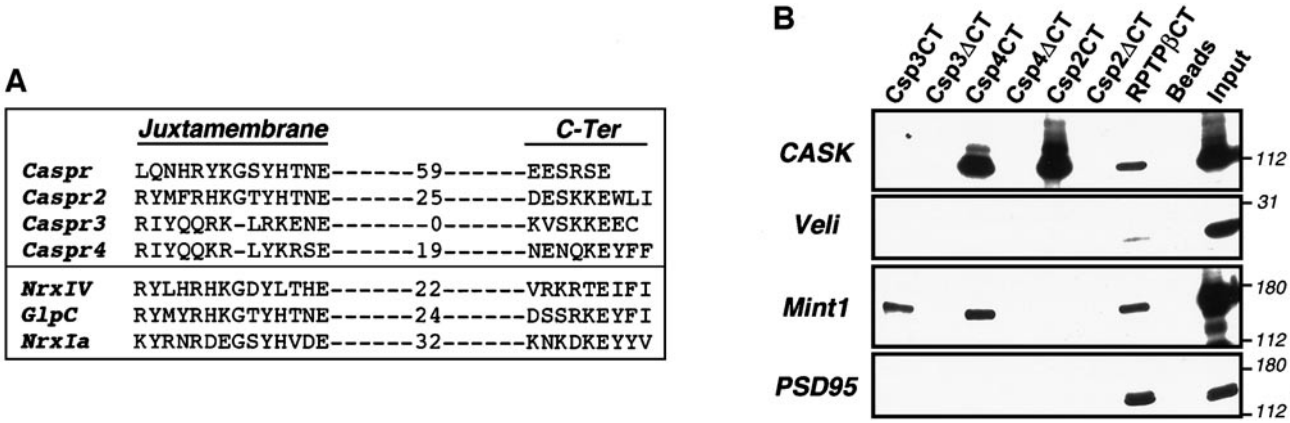

FIG. 7. Association of the cytoplasmic domains of Caspr proteins with PDZ domain-containing proteins. (A) Alignment of the amino acid sequence of the juxtamembrane and carboxy-terminal regions (C-Ter) found in cytoplasmic domains of the different human Caspr proteins, human Glycophorin C (GlpC), *Drosophila* neurexin-IV (NrxIV), and rat neurexin Iα (NrxIa). The juxtamembrane of Caspr and Caspr2 serves as a binding site for protein 4.1. A PDZ domain-binding site is found at the carboxy-terminus of Caspr2, Caspr3, and Caspr4. The number of amino acids that connect both regions is indicated for each sequence. (B) Interaction of the carboxy terminal tails of Caspr family proteins with PDZ domains. Lysates of HEK-293 cells expressing Myc-tagged CASK/Lin2, Veli/Lin7, Mint1/Lin 10, or PSD95 were mixed with the various indicated peptides immobilized to agarose beads. Peptide used included the carboxy terminal tails of Caspr4 (CSP4CT), Caspr3 (CSP3CT), Caspr2 (CSP2CT), receptor tyrosine phosphatase β/ζ (RPTPβCT), or similar peptides lacking the last amino acid (CSP2dCT, CSP3dCT, and CSP4dCT). Streptavidin agarose (Beads) were used as an additional negative control. Bound proteins were separated on SDS gels and blotted with an antibody to Myc-tag. While Caspr2 peptide precipitated CASK but not Mint1, Caspr3 interacted with Mint1 but not with CASK, and Caspr4 was able to bind both CASK and Mint1. A sample of the lysates used in the pulldown experiment is shown on the right of each panel (Input). Molecular weight markers are shown in kDa.

domain-containing molecule that binds to various transmembrane proteins (Irie *et al.*, 1997; Kawachi *et al.*, 1999; Sheng and Pak, 2000). This analysis demonstrates that the unique amino acid sequences found at the cytoplasmic tails of members of the Caspr family enables their interactions with distinct PDZ domains.

DISCUSSION

In this report we describe the molecular cloning and characterization of two novel members of the NCP family we termed Caspr3 and Caspr4. Caspr3 and Caspr4 represent two distinct genes that are localized to different human chromosomes. While Caspr3 was mapped to Chromosome 9q11, Caspr4 is located on chromosome 16q22 (GeneMap99 at NCBI). These sites are distinct from the known locations of Caspr on chromosome 17q21 (Peles *et al.*, 1997) and Caspr2 on chromosome 7q35-36 (Poliak *et al.*, 1999). Caspr3 and Caspr4 are transmembrane proteins displaying an overall structural similarity to the neurexins (Missler and Sudhof, 1998; Ushkaryov *et al.*, 1992). However, like the related Caspr and Caspr2 proteins (Menegoz *et al.*, 1997; Peles *et al.*, 1997; Poliak *et al.*, 1999), they are distinguished from the neurexins by the presence of a novel fibrinogen-like domain and a discoidin homology domain in their extracellular region (Fig. 1C). The latter is also found in Discoidin-I, a developmentally regulated lectin from Dictostylium discoideum that plays a role in cell adhesion (Springer *et al.*, 1984), and in coagulation factors V and VIII, where it mediates their attachment to phospholipids on the cell surface (Macedo-Ribeiro *et al.*, 1999). Alignment of the amino acid sequence of the extracellular domains of the different Caspr proteins reveals that Caspr3 and Caspr4 are more similar to each other (68% identity), then to Caspr2 (48–49% identity) or to Caspr (37–38%). It was previously demonstrated that the extracellular region of Caspr interacts laterally (i.e., in *cis*) with contactin, a GPI-linked cell adhesion molecule of the immunoglobulin superfamily, to create a complex on the cell surface (Peles *et al.*, 1997; Faivre-Sarrailh *et al.*, 2000; Rios *et al.*, 2000). In contrast, Caspr2, Caspr3, and Caspr4 do not bind to contactin (Poliak *et al.*, 1999; Gollan and Peles, unpublished observations), suggesting that despite their high structural similarity, the respective extracellular regions of the Caspr proteins mediate interactions with different ligands. Other members of the contactin subgroup, including TAG-1 (Furley *et al.*, 1990), BIG-1 and BIG-2 (Yoshihara *et al.*, 1994, 1995), NB2 and NB3 (Ogawa *et al.*, 1996), and FAR-2 (Plagge *et al.*, 2001), are all likely candidates to

form cell recognition complexes with Caspr2, Caspr3, and Caspr4.

In contrast to the other Caspr genes, several Caspr3 isoforms are generated by alternative splicing. We have cloned two forms of Caspr3 termed hCaspr3TM and hCaspr3S, which contain open reading frames of 1154 and 612 amino acids, respectively. Analysis of the human genome database (GenBank Accession Nos.: NT_027088 and NT_008389) revealed that the Caspr3 gene spans over 350 kb in length and contains 24 exons, most of which are around 180 bp long with the exception of the last exon which is 1.2 kb long, covering the last 200 bp of the coding sequence and all of the 3′UTR. Both Caspr3TM and Caspr3S lack exons 8–10, resulting in proteins that lack the first EGF domain and a small part of the fibrinogen-like region (Fig. 1C). Nevertheless, although exons 8–10 are missing from the cDNAs cloned here, they are found in many ESTs and in the partial sequence of KIAA1714 (Nagase *et al.,* 2000), making it likely that several Caspr3 isoforms exist as a result of alternative splicing. Furthermore, several other ESTs revealed the existence of additional alternative spliced forms of Caspr3 that are missing exons 14–15 (GenBank Accession No.: AW069086), 15 (GenBank Accession No.: AW242597), and 22 (GenBank Accession No.: BG779469). Finally, the Caspr3S transcript cloned from HS683 cells lacks also exons 13–24 and may represent a secreted isoform of Caspr3. RT-PCR analyses using specific primers to Caspr3TM or Caspr3S reveal the presence of both forms in corpus callosum, cortex and cerebellum from three different human brain tissues (data not shown), supporting the presence of Caspr3S in the normal nervous system. Further analyses using isoform-specific antibodies will be required to determine the existence of Caspr3S protein in the human brain.

Recent data suggest that NCP proteins may play a role in regulation of ion channel localization and function by various mechanisms (Bhat *et al.,* 2001; Boyle *et al.,* 2001; Poliak *et al.,* 1999, 2001; Yuan and Ganetzky, 1999). For example, it was shown that K^+ channels are mislocalized at the paranodes in contactin and Caspr knockout mice, suggesting that Caspr may function as a fence to separate the Na^+ channels at the nodes of Ranvier from the juxtaparanodal K^+ channels (Bhat *et al.,* 2001; Boyle *et al.,* 2001). It was suggested that Caspr2 is also involved in the proper placement of ion channels along the axon, albeit through a different mechanism that involves its coupling to the channels by an intermediate PDZ-domain scaffolding protein (Peles and Salzer, 2000; Poliak *et al.,* 1999). In this regard, Caspr2 function resembles that of the neurexin-ligand neuroligin, which may organize the postsynaptic membrane by coupling cell recognition with ion channel clustering through PSD95 (Song *et al.,* 1999). Evidence for a third mechanism by which NCPs may regulate the function of ion channels arose with the identification of axotactin in *Drosophila,* a secreted molecule produced by glia cells which affect nerve conduction (Yuan and Ganetzky, 1999). While the exact mechanism of axotactin function is unknown, it is possible that it regulates the clustering of ion channels on the axons (Yuan and Ganetzky, 1999). In analogy, clustering of Na^+ channels during the early phase of CNS myelination has also been shown to be regulated by a soluble factor secreted by oligodendrocytes (Kaplan *et al.,* 1997, 2001). We have found that Caspr3 is the only member of the NCPs expressed in oligodendrocytes (Figs. 3 and 4), raising the question of whether it may function similarly to axotactin in *Drosophila.* Interestingly, Caspr3 was not found in Schwann cells, which in contrast to oligodendrocytes regulate Na^+ channels clustering in the peripheral nervous system by direct contact with the axon (Ching *et al.,* 1999). Furthermore, although myelinating oligodendrocytes regulate axonal caliber, the rate of axonal transport and the local differentiation of the axon (de Waegh *et al.,* 1992), the molecules that mediate this communication are still largely unknown. The expression of both soluble and transmembrane forms of Caspr3 by oligodendrocytes makes this protein a good candidate for mediating glia-neuron interaction as suggested for Caspr and Caspr2.

We have found that the cytoplasmic domains of Caspr3, Caspr4, and Caspr2, interact with different PDZ domain-containing proteins known to participate in the organization of protein complexes at the plasma membrane (Fanning and Anderson, 1999). Such proteins have been implicated in the establishment of specialized membrane domains (Gonzalez-Mariscal *et al.,* 2000), clustering of transmembrane receptors (Sheng and Pak, 2000), membrane protein trafficking (Garner *et al.,* 2000), and the generation of multi-protein signaling complexes (Scott and Zuker, 1998). PDZ domains have been grouped into several classes depending on the sequence of the ligands they bind (Songyang *et al.,* 1997; Vaccaro *et al.,* 2001). Inspection of the carboxy-terminal tail regions of Caspr family members (Fig. 7A), reveals that Caspr4, Caspr2, *Drosophila* neurexin IV, and the neurexins share a carboxy-terminal motif containing hydrophobic or aromatic residues at the position P_0 and P_{-2} (C-terminus referred to as position 0), classified as a type II PDZ ligands (Songyang *et al.,* 1997; Vaccaro *et al.,* 2001). In contrast, the carboxy-terminal sequence of Caspr3 contains two acidic residues at position P_{-1} and P_{-2} and ends with a cysteine at the P_0 position, a sequence which may represent a novel binding site for type III PDZ domains (Maximov *et al.,* 1999; Vaccaro *et al.,* 2001). Consequently, Caspr2, and Caspr4, but not Caspr3, interact with CASK, which contains a single type II PDZ domain. Yet, while Caspr4 binds also to Mint1, Caspr2 was not,

indicating that although they belong to the same group of binding ligands, differences in their C-terminal sequence (KEWLI-COOH vs KEYFF-COOH, respectively) enable them to interact with distinct proteins. While Caspr3 did not interact with CASK, it binds Mint1 (Fig. 7B). Mint1 contains two PDZ domains of which one was shown to bind a C-terminal cysteine ligand (Maximov *et al.*, 1999; Biederer and Sudhof, 2000). Although we have not mapped the binding site for Caspr3 in Mint1, the observation that removal of the last cysteine from Caspr3 abolished the interactions, strongly suggests that it binds one of the two PDZ domains in Mint1. In *C. elegans,* Mint1 homologue Lin10 is found in a complex with Lin2 and Lin7 that controls the localization of the EGF receptor to the basolateral surface of the body wall epithelium (Kaech *et al.*, 1998), and is also important for the localization of glutamate receptors (Rongo *et al.*, 1998). In the mammalian brain, Mint1 is found in a tripartite complex with CASK/Lin2 and Veli/Lin7 that binds the neurexins, where they are thought to play a role in membrane organization and exocytosis (Biederer and Sudhof, 2000; Borg *et al.*, 1998; Butz *et al.*, 1998). In addition, this complex was shown to play a role in vesicle transport by bridging between kinesin motor proteins and their cargo (Setou *et al.*, 2000). Immunolocalization of Caspr3 revealed that its unique distribution along axons, raising the intriguing possibility that this protein may also be involved in vesicle transport along the axon. In analogy with the neurexins, binding of the Caspr proteins to this complex may generate a scaffolding center that coordinates the recruitment of other proteins important for cell–cell communication and membrane trafficking in the nervous system.

EXPERIMENTAL METHODS

Cloning and Expression

EST AI148152 was used as a probe to isolate human Caspr3 from a ZAP-Hs683 cDNA library. The same probe was used to isolate a 1.6kb fragment of Caspr4 from a mouse genomic DNA library (Stratagene, San Diego, CA). A 160-bp-long fragment highly homologous to exon 11 of hCaspr3 was generated by PCR (5′ primer: GAT GGA ACC TCC CTG AGC T; 3′ primer: CAT TCA TTC CGG TCA GCA TCA C) and was further used to clone the full-length mouse Caspr4 from a fetal mouse brain cDNA library (Stratagene, San Diego, CA). The sequences of both Caspr3 and Caspr4 cDNAs were determined on both strands by priming with synthetic oligonucleotides (GenBank Accession Nos.: AF333769 and AF333770). The sequence of human Caspr3 shown in Fig. 1 contains exons 9–11 that are found in KIAA1714 but not in hCaspr3TM. Expression constructs were made by subcloning a 4-kb fragment containing the full-length hCaspr3TM or a 4.5-kb containing the full-length mCaspr4 into pCDNA3 (Invitrogen) to generate pC3-hCaspr3TM and pC3-mCaspr4, respectively. Generation of Caspr and Caspr2 expression constructs pC3-hCaspr and pC3-hCaspr2 were described previously (Peles *et al.*, 1997; Poliak *et al.*, 1999). Myc-tagged versions of mouse Lin2 (CASK), mouse Lin7 (Veli/MALS) and human X11α (Mint1/Lin10) were kindly obtained from Ben Margolis (Ann Aarbor, Michigan) (Borg *et al.*, 1998). The PSD95-Myc construct was made by replacing the first eight amino acids of rat PSD95 with the tag.

Northern Blot Analysis

DNA fragments of 0.5, 2.5, or 0.23 kb corresponding to positions 1442–2250, 967–3469, and 2543–2715 of the human *caspr3,* mouse *caspr4,* and human *caspr4* cDNAs, respectively, were used as probes for Northern blot analyses. The specificity of each probe for only one member of the Caspr family was examined by hybridization to filters containing serial dilutions of all four members of the family. Probes for Caspr and Caspr2 were described previously (Poliak *et al.*, 1999). DNA fragments were isolated, labeled by random priming, purified on a Sepharose G-50 column (Amersham-Pharmacia) and used as probes. Hybridization to multiple tissues Northern blots (MTN Blots, Clontech) was carried out as described previously (Peles *et al.*, 1997).

Preparation of Tissue Specimens

For analysis of human Caspr3, brain, spinal cord, and femoral nerve tissues were obtained from autopsies without neurological disease as approved by the ethics committee of the Department of Pathology (University of Basel). Post mortem times were between 4 and 30 h and patients ages were between 46 and 86 years. Fresh tissues were frozen on dry ice rapidly after dissection. For *in situ* hybridization of Caspr4, mice were anaesthetized and decapitated, and the tissues were rapidly dissected, embedded in O.C.T. compound (Miles, Elkhart, IN) and snap frozen in liquid nitrogen. For immunohistochemistry of Caspr4, mice were perfused with 2.5% paraformaldehyde (PFA) in PBS. Brains were dissected and incubated for several days in a solution of 1% PFA and 25% sucrose before freezing.

In Situ Hybridization

Synthetic digoxigenin-labeled riboprobes (cRNA) were generated from recombinant pBluescript plasmid

containing a cDNA insert of Caspr3TM, a 2.5-kb *Eco*RI-fragment of mouse Caspr4, rat neuron specific enolase (NSE, Schaeren-Wiemers *et al.*, 1997) and a 2.5-kb *Eco*RI / *Hin*dII fragment of PLP, respectively. Specificity of the probes was determined as described above for the Northern analyses. Transcription was done from both sides with either T3 or T7 RNA polymerase, generating antisense or sense (control) cRNA probes. The labeled cRNA probes were alkaline hydrolyzed to an average length of 200–400 bases. *In situ* hybridization was performed on cryosections of freshly frozen tissue as described previously (Schaeren-Wiemers and Gerfin-Moser, 1993).

Generation of Antibodies

Polyclonal antibodies against Caspr3TM were generated by immunizing rabbits with a synthetic peptide (QQRKLRKENESKVSKKEEC corresponding to amino acid residues 1136–1154) coupled to keyhole limpet hemocyanin (Ab 93–94). Antibodies were affinity purified on a column of the peptide antigens covalently coupled to agarose beads (Pierce, Rockford, IL). Antibodies against Caspr4 were generated by immunizing rabbits with a GST-fusion protein containing the cytoplasmic domain of mouse Caspr4 (Ab 85–86). Affinity purification was performed by first removing the antibodies against GST by passing the anti-serum through a column of Sepharose-GST (Pierce, Rockford, IL) and then on a column of GST-Caspr4CT Sepharose (Amersham-Pharmacia). Antibodies against Caspr and Caspr2 were previously described (Peles *et al.*, 1997; Poliak *et al.*, 1999).

Immunohistochemistry

For immunohistochemical analysis of Caspr3, cryostat sections (10–15 μm) of fresh frozen human tissues were mounted on gelatin chromalaun coated slides and fixed for 1 h in 10% buffered formalin solution or in 4% PFA, followed by three washes in PBS. Permeabilization was done overnight at room temperature in 50% ethanol. Endogenous peroxidase activity was blocked by incubation with 0.6% H_2O_2 + 80% Methanol in H_2O for 20 min at room temperature. Unspecific binding sites were blocked by incubation with 2.5% normal goat serum, 0.1% fish skin gelatin, 0.05% Saponin in PBS for 45 min at room temperature. Primary antibodies were incubated overnight at 4°C in blocking solution. Bound antibodies were detected using Vector Elite ABC-Kit (Vector Laboratories). Color reaction was performed with the peroxidase substrate AEC (3-amino-9-ethylcarbazole, 0.027% w / v; H_2O_2 0.03% v / v) in 0.1 M sodium acetate buffer pH 5.2 for 12 min at room temperature in the dark. Duplicate sections were weakly counter stained with Mayer's Haemalaun (Merck) for 1 min. Sections were embedded in Kaiser's glycerin gelatin (Merck). For Caspr4, staining was done using 16–32 μm floating sections as described previously (Poliak *et al.*, 1999).

Immunoprecipitation, Peptide Pulldown Experiments

Immunoprecipitation and immunoblotting analyses of transfected HEK-293 cells and rat brain membrane lysates were done essentially as described previously (Peles *et al.*, 1997; Poliak *et al.*, 1999). For pulldown experiments, 293 cells were transfected with myc-tagged mouse Lin2, mouse Lin7, human X11α, or rat PSD95 (Borg *et al.*, 1998). Cells were solubilized in TNTG (20 mM Tris 7.5, 0.1% Triton X-100, 150 mM NaCl, 10% glycerol, and protease inhibitors), and the lysates were incubated with 20 μg of biotinylated peptides coupled to Neutravidin beads (Pierce). Bound proteins were washes three times with TNTG, separated on SDS gels, and immunoblotted with anti-myc antibody (Poliak *et al.*, 1999).

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