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Regulated Expression and Subcellular Localization of Syndecan Heparan Sulfate Proteoglycans and the Syndecan-Binding Protein CASK/LIN-2 during Rat Brain Development

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The syndecan family of cell surface heparan sulfate proteoglycans interacts via their cytoplasmic C-terminal tail with the PDZ domain of CASK/LIN-2, a membrane-associated guanylate kinase homolog. The syndecan–CASK interaction may be involved in intercellular signaling and/or cell adhesion. Here we show that syndecan-1 to syndecan-4 have distinctive mRNA distributions in adult rat brain by *in situ* hybridization, with syndecan-2 and -3 being the major syndecans expressed in neurons of the forebrain. At the protein level, syndecan-2 and -3 are differentially localized within neurons; syndecan-3 is concentrated in axons, whereas syndecan-2 is localized in synapses. The synaptic accumulation of syndecan-2 occurs late in synapse development. CASK is a cytoplasmic-binding partner for syndecans, and its subcellular distribution changes strikingly during development, shifting from a primarily axonal distribution in the first 2 postnatal weeks to a somatodendritic distribution in adult brain. This change in CASK distribution correlates temporally and spatially with the expression patterns of syndecan-3 and -2, consistent with the association of both of these syndecans with CASK *in vivo*. In support of this, we were able to coimmunoprecipitate a complex of CASK and syndecan-3 from brain extracts. Our results indicate that specific syndecans are differentially expressed in various cell types of the brain and are targeted to distinct subcellular compartments in neurons, where they may serve specialized functions. Moreover, CASK is appropriately expressed and localized to interact with both syndecan-2 and -3 in different compartments of the neuron throughout postnatal development.

Key words: syndecan; CASK/LIN-2; heparan sulfate proteoglycan; MAGUK; subcellular targeting; axon

The syndecan family of transmembrane proteins comprises a major class of cell surface heparan sulfate proteoglycans (HSPGs). Currently, four members have been identified in mammals: syndecan-1, syndecan-2/fibroglycan, syndecan-3/N-syndecan, and syndecan-4/ryudecan (for review, see Bernfield et al., 1992; David, 1993; Carey, 1997). The extracellular functions of syndecans are primarily mediated by their heparan sulfate (HS) glycosaminoglycan (GAG) side chains, which have affinity for a wide variety of secreted molecules and extracellular matrix (ECM) components. Recent interest in cell surface HSPGs stems particularly from the discovery that they are important for the binding and action of polypeptide growth factors, as well as for cell–matrix and cell–cell interactions (for review, see Bernfield et al., 1992; David, 1993; Schlessinger et al., 1995; Couchman and Woods, 1996; Carey, 1997;). In this context, cell surface HSPGs such as syndecans can be regarded as "coreceptors" that function to present or concentrate polypeptide factors for their specific high-affinity receptor tyrosine kinases (for review, see Schlessinger et al., 1995; Carey, 1997).

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In addition to binding to polypeptide signaling factors, HS also binds to ECM components including fibronectin, collagen, and laminin and to the cell surface protein neural CAM. Many studies have implicated cell surface HSPGs in cell adhesion and tissue morphogenesis (for review, see Bernfield et al., 1992; David, 1993; Carey, 1997). On the basis of their ability to mediate dynamic cell–matrix interactions and to bind to diverse polypeptide signaling factors, syndecan HSPGs could play a supporting role in many aspects of neural development, including cell migration, neurite extension, and synapse assembly and plasticity. We have recently discovered that syndecan-2 is concentrated in synaptic junctions of adult rat brain (Hsueh et al., 1998). This has been confirmed by Ethell and Yamaguchi (1999) who additionally showed that overexpression of syndecan-2 in neurons alters the morphological development of dendritic spines. However, not much is known about which other syndecans are specifically expressed in neurons, how they are distributed at the subcellular level, and how their expression patterns are regulated during development of the nervous system.

CASK, the mammalian homolog of *Caenorhabditis elegans* LIN-2, is a protein of the membrane-associated guanylate kinase homolog (MAGUK) superfamily. It contains a Ca^{2+}/calmodulin-dependent kinase-like domain at its N terminal, in addition to a PDZ domain, an SH3 domain, and a guanylate kinase-like domain that characterizes all MAGUK proteins (Hata et al., 1996; Hoskins et al., 1996; Dimitratos et al., 1997). In *C. elegans*, LIN-2 together with LIN-7 and LIN-10 (two other PDZ proteins) forms a ternary protein complex that plays a critical role in basolateral localization of LET-23, an epidermal growth factor receptor homolog, in epithelial cells (Kaech et al.,

1998). A homologous ternary protein complex has been demonstrated in mammalian brain, although the functional significance of the mammalian LIN-2/LIN-7/LIN-10 complex remains to be determined (Butz et al., 1998). In addition to binding to LIN-7 and LIN-10 homologs, CASK has been shown to interact via its PDZ domain with the C terminals of neurexins (Hata et al., 1996) and syndecans (Cohen et al., 1998; Hsueh et al., 1998). Neurexins and syndecans are transmembrane proteins that share a similar cytoplasmic C-terminal sequence (-EYYV and -EFYA, respectively) and a common involvement in cell–cell or cell–ECM interactions (for review, see Missler et al., 1998). The intracellular interaction with CASK, or other PDZ proteins such as syntenin (Grootjans et al., 1997), may link neurexins and syndecans to the cytoskeleton (Cohen et al., 1998) or to intracellular signaling pathways.

The four mammalian syndecans (as well as *Drosophila* and *C. elegans* syndecans) share the same -EFYA C-terminal motif that mediates binding to the PDZ domain of CASK (Hsueh et al., 1998), suggesting that CASK might associate with all syndecan members *in vivo*. We have recently provided immunohistochemical evidence of an interaction between CASK and syndecan-2 in synaptic junctions of adult brain (Hsueh et al., 1998). At the subcellular level, however, CASK is more widely distributed than the synaptically localized syndecan-2 (Hsueh et al., 1998), raising the possibility that CASK associates with other syndecans in nonsynaptic regions of the neuron. However, little is known about the expression patterns of the different syndecans in relation to CASK or how these relationships change during development of the rat brain.

To characterize the extent of CASK–syndecan interactions *in vivo*, we investigated the expression patterns of CASK and of the different syndecans in developing and mature rat brain. We focused on syndecan-2 and -3 after determining that they were the major neuronal syndecans of the forebrain. Syndecan-2 and -3 show contrasting expression profiles during development and segregated distribution within neurons. Syndecan-3 is more highly expressed in developing brain and concentrated in axons. Syndecan-2 is more strongly expressed in mature brain and localized in synapses. CASK protein levels are high throughout development, but its distribution changes from an axonal pattern (where it colocalizes with syndecan-3) to a somatodendritic pattern (where it overlaps with syndecan-2). These findings are consistent with the binding of syndecan-2 and -3 to CASK at different times in development and in different compartments of the neuron. In support of this biochemical association, we report for the first time the coimmunoprecipitation of CASK and syndecan from brain extracts.

MATERIALS AND METHODS

In situ *hybridization.* ^{35}S-labeled RNA probes were synthesized by *in vitro* transcription using T7 and SP6 RNA polymerase. The transcription templates were generated by PCR and corresponded to rat syndecan-1 [nucleotide (nt) +90 to +615], syndecan-2 (nt −22 to +454), syndecan-3 (nt +143 to +626), and syndecan-4 (nt +1 to +414). These templates represent the poorly conserved ectodomains of each syndecan. T7 and SP6 promoters were included in antisense and sense PCR primers, respectively.

Antibodies. Rabbit polyclonal syndecan-2 peptide antibodies (Syn-2C) and CASK peptide antibodies have been described (Hsueh et al., 1998). CASK murine monoclonal antibodies K56A/50.1, K56A/57.1, and K56A/95.1 were raised against a glutathione *S*-transferase fusion containing amino acids 317–415 of rat CASK (Cohen et al., 1998) in collaboration with Dr. James Trimmer (State University of New York, Stony Brook, NY). These three monoclonal antibodies are specific for CASK and give identical results on immunoblotting and immunostaining (Y.-P. Hsueh, unpublished observations). Syndecan-3 cytoplasmic domain antibodies (Syn-3C) were raised in rabbits against the synthetic peptide SYTLEEPKQASVTYQK. Rabbit polyclonal antibodies against the ectodomain of syndecan-3 (Syn-3ecto) were a gift of Dr. David Carey (Geisinger Clinic, Danville, PA). Murine monoclonals to MAP2, synaptophysin, and calbindin-D were purchased from Sigma (St. Louis, MO).

DNA constructs. Rat syndecan-2 cDNA was generously provided by Dr. Graham Cowling (Manchester University, Manchester, UK) and subcloned into the *Kpn*I and *Eco*RI sites of mammalian expression vector GW1-CMV. Syndecan-3 cDNA was kindly provided by Dr. David Carey and subcloned into GW1-CMV. The coding sequences of syndecan-1 and -4 were amplified from rat brain mRNA by reverse transcription-PCR and subcloned into the *Kpn*I and *Eco*RI sites of GW1-CMV.

Transfection, immunocytochemistry, and immunohistochemistry. Transfection of COS-7 cells using lipofectamine and subsequent immunocytochemistry were performed as described (Hsueh and Sheng, 1999). For rat brain immunohistochemistry, floating sections at different ages were prepared and processed as described (Hsueh et al., 1998) with slight modifications. After perfusion and fixation, postnatal day 21 (P21) rat brains were directly sliced with a vibratome, whereas P3, P7, and P15 rat brains were embedded in 2–3% agarose before slicing on the vibratome at 50 μm thickness. For immunostaining using Syn-3ecto antibodies, tyramide signal amplification (TSA direct kit; NEN Life Science Products, Boston, MA) was applied according to the manufacturer's directions to conserve antibody and enhance signal. The staining pattern using the TSA kit was identical to that using standard indirect immunofluorescence methods. Fluorescence was viewed with a Zeiss Axioskop microscope or a Bio-Rad (MRC-1000; Hercules, CA) confocal microscope. Images were prepared for publication with Adobe PhotoShop.

Immunoprecipitation and immunoblotting for HSPG syndecans. For immunoprecipitation from rat brain extracts, 10 μg of affinity-purified Syn-3C antibodies was incubated with 60 μl of a 1:1 slurry of protein A Sepharose in 100 mM sodium borate buffer, pH 8.0, at 4°C overnight. Free antibodies were removed by washing with sodium borate buffer three times. These antibody–protein A Sepharose pellets were used for immunoprecipitation. One-week-old rat brains were minced and homogenized with a Polytron in PBS. The extract was cleared of nuclei, unbroken cells, etc., by centrifugation at 930 × *g* for 10 min at 4°C. The postnuclear supernatant was solubilized with 1% Triton X-100 in PBS at 4°C for 1 hr and dialyzed against 0.1% Triton X-100 in PBS at 4°C overnight. Insoluble material was pelleted by centrifugation at 37,000 × *g* for 1 hr. Detergent-solubilized extract (1 mg of protein) was incubated with antibody–protein A Sepharose at 4°C for 3–4 hr. The precipitates were washed with 0.1% Triton X-100 in PBS three times and with 10 mM Tris, pH 7.5, once. Bound proteins were eluted from beads in SDS sample buffer and immunoblotted as described (Kim et al., 1994). Briefly, proteins were separated on a TBE/urea 3–15% polyacrylamide gradient gel (40 mM Tris, 60 mM boric acid, 0.8 mM EDTA, 1 mM sodium sulfate, and 2.7 M urea; 7.5% cross-linking) with TBE running buffer containing 0.1% SDS and were transferred onto Immobilon-N (Millipore, Bedford, MA).

Primary cultures of rat cortical neurons. After trypsinization and mechanical dissociation, cortical neurons from embryonic day 16 (E16)–E17 rat embryos were resuspended in DMEM supplemented with 10% FCS and 10% horse serum and plated on coverslips coated with poly-L-lysine (1 mg/ml) at densities of 2–4 × 10^5 per 18 mm coverslip. Immunofluorescence staining was performed at 4–7 d *in vitro.*

Subcellular fractionation of rat brain extracts. Subcellular fractions of adult rat brain were prepared as described (Huttner et al., 1983). Briefly, rat brain Dounce homogenate was centrifuged at 1000 × *g* to remove nuclei and other large debris [first pellet (P1)]. The supernatant was centrifuged at 10,000 × *g* to obtain a crude synaptosomal fraction (P2), which was subsequently lysed with hypotonic buffer and centrifuged at 25,000 × *g* to pellet a lysed synaptosomal membrane fraction (LP1). The supernatant (LS1) was then centrifuged at 165,000 × *g* to obtain a crude synaptic vesicle fraction (LP2) and soluble fraction (LS2). The supernatant above the P2 fraction (S2) was centrifuged at 165,000 × *g* to obtain a soluble fraction (S3) and a light membrane fraction (P3). For developmental studies, P2 fractions were collected from the rats at the ages of E14, E18, P3, P7, P15, P22, and P42.

RESULTS

Differential expression of syndecan mRNAs in rat brain

To identify which syndecans might interact with CASK in neurons *in vivo*, we analyzed by *in situ* hybridization (ISH) the

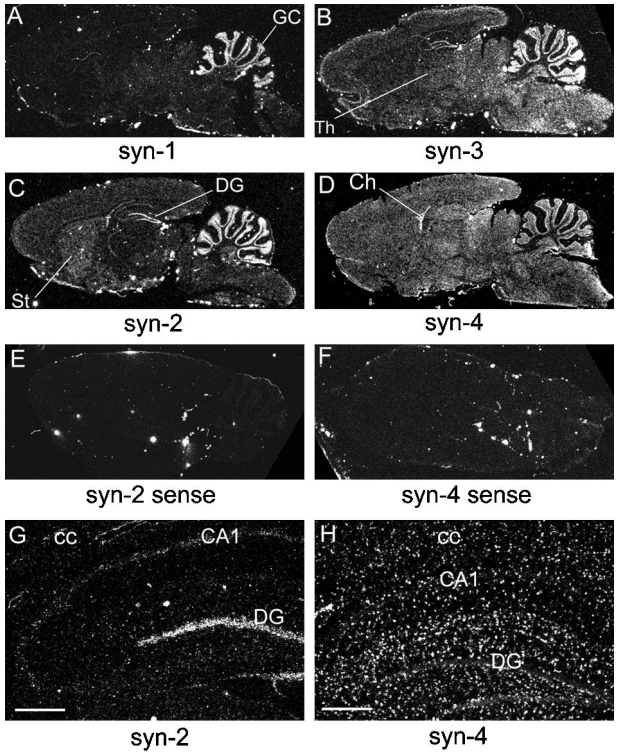

Figure 1. *In situ* hybridization analysis of syndecan mRNAs in adult rat brain. *A–D*, Syndecan-1 (*A*); syndecan-3 (*B*); syndecan-2 (*C*); syndecan-4 (*D*). *E, F,* Sense strand negative controls for syndecan-2 (*E*) and syndecan-4 (*F*). Syndecan-1 and -3 sense stand negative controls also gave no significant ISH signal (data not shown). *G*, *H*, Higher magnification (emulsion autoradiography) views of syndecan-2 (*G*) and syndecan-4 (*H*) distribution in the hippocampus, showing glial expression of syndecan-4. *cc*, Corpus callosum; *Ch*, choroid plexus; *DG*, dentate gyrus; *GC*, granule cell layer of the cerebellum; *St*, striatum; *syn*, syndecan; *Th*, thalamus. Scale bars, 400 μm.

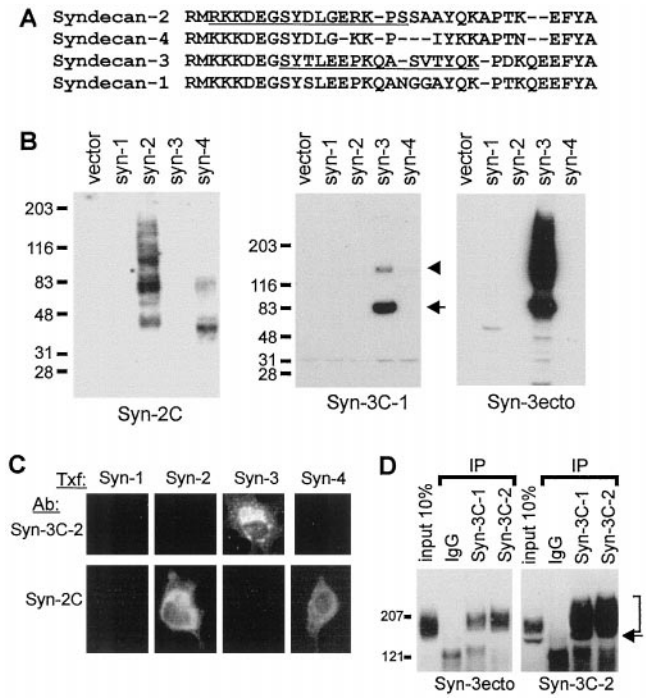

Figure 2. Characterization of Syn-2C and Syn-3C antibodies. *A,* Amino acid alignment of the cytoplasmic C-terminal tails of syndecan-1 to -4 is shown. Syn-2C and Syn-3C antibodies were raised against the *underlined peptide sequences* in the cytoplasmic domains of syndecan-2 and -3, respectively. *Dashes* represent gaps in the sequence introduced to maximize alignment of conserved sequences. *B,* Specificity of Syn-2C and Syn-3C antibodies was tested by immunoblotting using COS-7 cells transfected with syndecan-1, -2, -3, or -4 or vector alone, as indicated. Syn-2C recognizes heterologously expressed syndecan-2 and cross-reacts with syndecan-4. Syn-3C-1 specifically recognizes two protein bands (~83 kDa, *arrow*; ~130 kDa, *arrowhead*) in COS-7 cells transfected with syndecan-3 but not other syndecans. Similar results were obtained with Syn-3C-2 antibodies (data not shown). Syn-3ecto antibodies also recognize the ~83 kDa band, as well as a broad high–molecular weight smear that likely represents more fully modified, cleaved forms of syndecan-3. *C,* Specificity of Syn-2C and Syn-3C antibodies on immunofluorescence staining of COS cells transfected with syndecan-1, -2, -3, or -4, as indicated, is shown. *D,* Syn-3C antibodies immunoprecipitate syndecan-3 from rat brain extracts. Immunoprecipitation with affinity-purified Syn-3C antibodies (*Syn-3C-1* and *Syn-3C-2* are from different rabbits) was performed using P8 rat brain extracts. Immunoprecipitates were immunoblotted with Syn-3ecto or Syn-3C antibodies, as indicated. Control immunoprecipitations were performed with nonimmune purified rabbit IgGs (*IgG*). The ~150 kDa band is indicated by an *arrow*, and the 150–250 kDa smear is indicated by the *bracket*. An *input lane* contains 10% of the detergent extract used for immunoprecipitation (*IP*).

expression patterns of the four known syndecans. Each of the syndecans has a different distribution in the adult rat brain (Fig. 1). Syndecan-1 mRNA is expressed almost exclusively in the cerebellum (Fig. 1*A*); no signal was detected in the forebrain. Syndecan-2 is widely expressed, with high levels in the dentate gyrus of the hippocampal formation and in the granule cell layer of the cerebellum, moderate levels in the striatum and cerebral cortex, and low levels in the thalamus (Fig. 1*C*). Syndecan-3 is expressed strongly in granule cells and Purkinje cells of the cerebellum and moderately in the hippocampal formation, cerebral cortex, and thalamus (Fig. 1*B;* data not shown). Syndecan-4 mRNA is distributed in a diffuse cellular pattern throughout the rat brain, including white matter regions (Fig. 1*D*), suggesting that it is expressed specifically in glial cells. This is more obvious by emulsion autoradiography, which shows a pattern of syndecan-4 expression typical of astrocytes (hippocampal region is shown in Fig. 1*H*). Syndecan-4 also exhibits a glial pattern of ISH in other parts of the brain (data not shown).

In contrast to that for syndecan-4, the ISH signal for syndecan-2 and -3 in the hippocampus is concentrated over neuronal cell bodies of the dentate granule cell layer and the CA1/CA3 pyramidal cell layers (Fig. 1*G;* data not shown). Thus both syndecan-2 and -3 are expressed predominantly in neurons. In summary, these ISH studies reveal syndecan-2 and -3 to be the major neuronal syndecans of the forebrain, whereas syndecan-4 is expressed specifically in glial cells. Because of our particular interest in the functions and interactions of syndecans in neurons of the forebrain, we focus in this report on syndecan-2 and -3.

Specificity of syndecan-2 and -3 antibodies

To study syndecan-2 and -3 at the protein level, we raised rabbit polyclonal antibodies against specific peptide sequences in the intracellular C-terminal tail of syndecan-2 and -3 (Fig. 2*A*). By immunoblotting and by immunocytochemistry, Syn-2C antibodies were able to recognize syndecan-2 expressed heterologously in COS-7 cells (Fig. 2*B*,*C*). As is typical for HSPGs, heterologously expressed syndecan-2 ran on immunoblots as a highly heterogeneous smear of bands, ranging in apparent size from ~40 to ~200 kDa on TBE/urea gels (Fig. 2*B*). Syn-2C antibodies showed significant cross-reactivity with syndecan-4 but not syndecan-1 or -3 (Fig. 2*B*,*C*). This is not surprising because the cytoplasmic tails

of syndecan-2 and -4 are the most closely related within the syndecan family (see Fig. 2*A*).

For syndecan-3, two rabbit antisera raised against the same peptide (termed Syn-3C-1 and Syn-3C-2) gave essentially identical results (Fig. 2; data not shown). Neither of these Syn-C antibodies showed reactivity against syndecan-1, -2, or -4, but they did specifically recognize two bands on Western blots of syndecan-3–transfected COS-7 cells (a major band of ~83 kDa; a minor band of ~130 kDa; Fig. 2*B*). Syn-3C antibodies were also specific for syndecan-3 by immunofluorescence staining (Fig. 2*C*).

Interestingly, the pattern of Western bands recognized by Syn-3C (directed against the cytoplasmic tail of syndecan-3) differed from that seen by Syn-3ecto antibodies [which are specific for an extracellular region of syndecan-3 (Carey et al., 1992)]. The Syn-3ecto antibodies recognized the ~83 kDa band in common with Syn-3C antibodies but in addition revealed a strong smear of syndecan-3 immunoreactivity that extended well beyond ~200 kDa (Fig. 2*B*, compare *middle* with *right panels*). The heterogeneity of bands seen by the Syn-3ecto antibodies is typical of HSPGs on protein gels. The simplest explanation for the difference between the immunoblotting patterns of Syn-3C and Syn-3ecto antibodies is that the majority of the higher molecular weight species (the smear) consists of syndecan-3 that has lost its short cytoplasmic tail. Such an interpretation is consistent with the known extracellular cleavage of syndecan HSPGs, which occurs at a site close to the membrane in both cultured cells and *in vivo* (Carey et al., 1997; Subramanian et al., 1997). The ~83 kDa band presumably represents an "immature" form of syndecan-3 that has been partially modified but not yet cleaved, thus maintaining the connection between cytoplasmic and ectodomains.

To explore this issue further *in vivo*, we reasoned that unless all of syndecan-3 in the brain is cleaved at the juxtamembrane site, Syn-3C antibodies should immunoprecipitate a subset of endogenous syndecan-3 that should also be recognized by Syn-3ecto antibodies. Indeed, Syn-3C immunoprecipitates from brain extracts at P8 contained a high–molecular weight smear that was reactive for Syn-3ecto antibodies (Fig. 2*D*, *left*). This result indicates that uncleaved ("intact") syndecan-3 is present in these brain extracts; in addition, it provides further evidence of the correct specificity of both syndecan-3 antibodies. As expected, Syn-3C immunoprecipitates contained a high–molecular weight smear that was recognized by Syn-3C antibodies (Fig. 2*D*, *right*). Significantly, however, the Syn-3C antibodies precipitated only ~5% of the Syn-3ecto immunoreactivity from the extract, whereas in the same reaction, ~30–50% of the Syn-3C immunoreactivity was precipitated (Fig. 2*D,* compare *left* with *right panels*). If syndecan-3 was completely intact, equal fractions of intracellular and extracellular domains should coprecipitate; our finding therefore implies that the Syn-3ecto epitope is associated with the Syn-3C epitope with a stoichiometry of much less than one. The simplest explanation of these results is that a majority of syndecan-3 in the brain is cleaved at the juxtamembrane site, thereby separating the ectodomain from the cytoplasmic domain. Note also that Syn-3C antibodies recognize a lower band of syndecan-3 (~150 kDa) in the brain more prominently than do Syn-3ecto antibodies (Fig. 2*D, arrow*). This band likely represents an immature intact syndecan-3, which constitutes a larger proportion of the Syn-3C–immunoreactive pool than of the Syn-3ecto–immunoreactive pool. With postnatal maturation of the brain, this ~150 kDa band becomes increasingly prominent relative to the higher molecular weight smear on Syn-3C immunoblots (data not shown; see Fig. 3), suggesting that the higher molecular weight forms of syndecan-3 are more completely cleaved as brain development progresses. In summary, brain syndecan-3 runs as a high–molecular weight smear, as expected for a HSPG; and the majority of high–molecular weight syndecan-3 lack the cytoplasmic domain, presumably because of juxtamembrane cleavage of the protein.

Differential biochemical fractionation of cytoplasmic and extracellular epitopes of syndecan-3 in brain

We investigated the subcellular distribution of syndecan-3 protein by biochemical fractionation of brain homogenates. Interestingly, the fractionation profile of bands recognized by Syn-3C was very different from that recognized by Syn-3ecto antibodies (Fig. 3). In adult brain, Syn-3C recognizes predominantly the ~150 kDa band noted previously, whereas Syn-3ecto labels predominantly a broad smear of M_r ~150–250 kDa (Fig. 3*A*). The ~150 kDa band must therefore contain the cytoplasmic domain of syndecan-3 and can be considered an immature uncleaved form of syndecan-3. As befits an intact transmembrane protein, this ~150 kDa Syn-3C–reactive band is found only in particulate or membrane fractions such as P1, P2, and LP1 and not in the soluble fraction S3. The bulk of this syndecan-3 species [which we will term "syndecan-3(C150)" because it contains the cytoplasmic tail and runs at ~150 kDa] is present in the P1 fraction, which is enriched in cell soma, nuclei, and nuclei-associated membranes (Fig. 3). In contrast, the Syn-3ecto–reactive smear of ~150–250 kDa (which we will term "syndecan-3ecto") purifies predominantly into the microsome-enriched fraction (P3) and the soluble fraction (S3) of brain. (S2 also contains abundant syndecan-3ecto, but this is the parent fraction from which S3 and P3 are derived). The solubility of the ~150–250 kDa smear recognized by Syn-3ecto but not by Syn-3C antibodies is entirely consistent with this set of polypeptides being extracellularly cleaved syndecan-3 that has been "shed" from the membrane without its transmembrane and cytoplasmic domains. This shedding need not be from the cell surface but could also be occurring within the lumen of intracellular membrane compartments. Such a possibility is consistent with the abundance of the Syn-3ecto–reactive smear in P3 (microsome-enriched fraction) and in LP2 (a synaptic vesicle–enriched fraction that is isolated after hypotonic lysis of the P2 synaptosomal fraction). The differential fractionation of Syn-3C– and Syn-3ecto–immunoreactive bands in the brain extends the COS cell results, strongly supporting the idea that the majority of endogenous syndecan-3 is cleaved and shed from the membrane *in vivo*.

Perhaps because of the low sensitivity of these antibodies and/or the low expression levels of endogenous syndecan-2, our Syn-2C antibodies were unable to detect signals by Western blotting of rat brain, despite being able to recognize syndecan-2 overexpressed in COS cells. Another possibility is that the Syn-2C antibodies recognize a folded conformation of syndecan-2 that is destroyed by SDS-PAGE. Thus we were unable to investigate fractionation behavior and shedding of syndecan-2 *in vivo*. CASK, a cytoplasmic protein, is widely distributed among membrane and soluble fractions in rat brain (Fig. 3*A*).

In a previous study we were unable to show coimmunoprecipitation of CASK and syndecan-2 from brain extracts (Hsueh et al., 1998), probably because our Syn-2C antibodies were inadequate. With the better antibodies against syndecan-3, however, we were able to coimmunoprecipitate CASK with syndecan-3 antibodies (Fig. 3*B*). This coimmunoprecipitation was eliminated by preincubating the Syn3-C antibodies with the immunogen peptide and

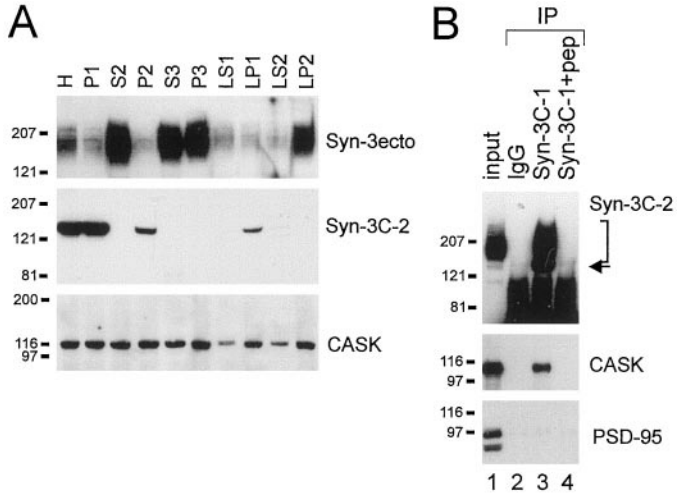

Figure 3. Subcellular fractionation and biochemical association of syndecan-3 and CASK in rat brain. *A,* Immunoblotting of subcellular fractions of rat brain with Syn-3C, Syn-3ecto, and CASK antibodies. *B,* Coimmunoprecipitation of CASK and syndecan-3 from P7 rat brain extracts by affinity-purified Syn-3C-1 antibodies. Negative control precipitations were performed with purified nonimmune rabbit IgG (*lane 2*) or after preincubation of Syn-3C-1 antibody with antigenic peptide (*pep*) (*lane 4*). *Input lanes* contain 10% (for Syn-3C immunoblot) or 5% (for CASK and PSD-95 immunoblots) of the detergent extract used for immunoprecipitation. Immunoprecipitates were probed for syndecan-3, CASK, and PSD-95. The *arrow* and *bracket* are described in Figure 2*D*. *H*, Total homogenate; other fractions are described in Materials and Methods.

is not seen with nonspecific IgGs, testifying to its specificity. Moreover, PSD-95 was not coprecipitated with syndecan-3 and CASK (Fig. 3*B*). These results confirm that CASK is associated with syndecan-3 in a protein complex *in vivo*.

Regional distribution of syndecan-3 in rat brain

We next used immunohistochemistry to examine the distribution of syndecan-3 in rat brain. Throughout postnatal development and in all regions of the brain, white matter tracts and axon pathways were the predominant structures stained by both Syn-3C and Syn-3ecto antibodies (Fig. 4; data not shown). A quantitative difference only was noticed in that syndecan-3 immunostaining is stronger at P3, P7, and P15 than in the adult, consistent with a higher level of syndecan-3 expression at early postnatal stages (Carey et al., 1997). In P7 rat brain, both Syn-3ecto and Syn-3C showed very similar fiber-like staining in axon tracts and in white matter, e.g., the corpus callosum (Fig. 4*D*,*E*), fimbria, and alveus of the hippocampus (Fig. 4*B*,*C*). These data suggest that syndecan-3 protein is concentrated in axons. However, significant differences were noted between the staining pattern of Syn-3C versus Syn-3ecto antibodies. In addition to the strong axonal and white matter staining, Syn-3C antibodies gave weaker signals that were not detected by Syn-3ecto antibodies: for instance, in the pyramidal neurons of the cerebral cortex (compare Fig. 4*D* vs *E*) and in the dentate gyrus and region CA3 of the hippocampus (Fig. 4*B* vs *C*). Overall, the Syn-3C immunohistochemistry pattern is consistent with the ISH results. Syn-3C labeling was virtually abolished by preincubation of the antibodies with the antigenic peptide (Fig. 4*F*), indicating the specificity of the Syn-3C immunostaining. The differences that exist between the Syn-3C– and Syn-3ecto–staining patterns may be accounted for by the shedding of the ectodomain of syndecan-3 after extensive cleavage of that occurs in the brain (see above). The cleaved syndecan-3 ectodomain may be relatively dispersed in the tissue or lost during the permeabilization and washing steps of the immunostaining procedure, hence giving rise to the reduced staining with Syn-3ecto antibodies. Nevertheless, staining with both Syn-3C and Syn-3ecto antibodies indicates that syndecan-3 is concentrated in axon tracts of the brain, in agreement with previous reports (Nolo et al., 1995; Carey et al., 1997).

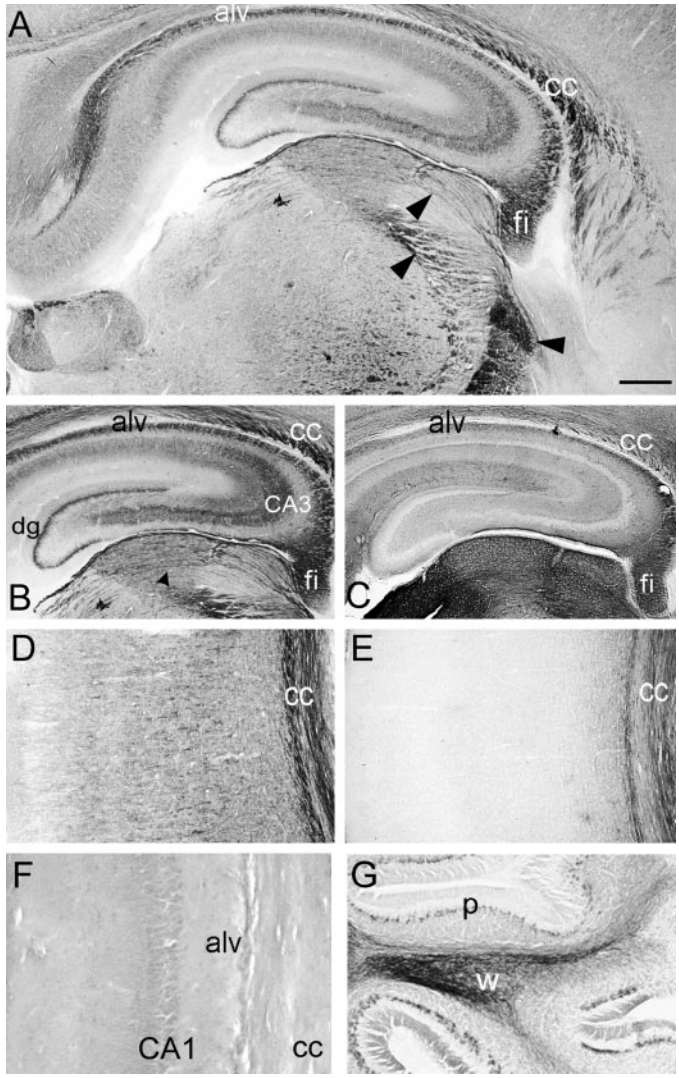

Figure 4. Localization of syndecan-3 in P7 rat brain by DAB immunohistochemistry. *A,* Syn-3C immunoreactivity is highly concentrated in axonal pathways [e.g., the fimbria (*fi*) and alveus (*alv*) of the hippocampal formation], the *cc*, and axon tracts running through the thalamus (*arrowheads*). *B–E*, Staining patterns of antibodies Syn-3C (*B, D*) and Syn-3ecto (*C, E*) are compared in the hippocampal formation (*B, C*) and cerebral cortex (*D, E*). *F,* Preincubation with Syn-3C antigenic peptide blocks the Syn-3C immunostaining of the corpus callosum and alveus; weak background staining of cell bodies of CA1 remains. *G,* Syn-3C antibodies strongly label the white matter (*w*) of the cerebellum. *dg*, Dentate gyrus; *P*, Purkinje cell layer. Scale bar: *A,* 400 μm; *B-–E*, *G,* 350 μm; *F,* 150 μm.

Confocal immunofluorescence microscopy confirmed the association of syndecan-3 with axons (Fig. 5). Bright syndecan-3 staining was seen on axons of the mossy fiber tract (Fig. 5*A*), axons of the corpus callosum, stria terminalis, and cerebellar white matter (Fig. 5*B–D*), and axons of cerebellar basket cells (Fig. 5*E1*).

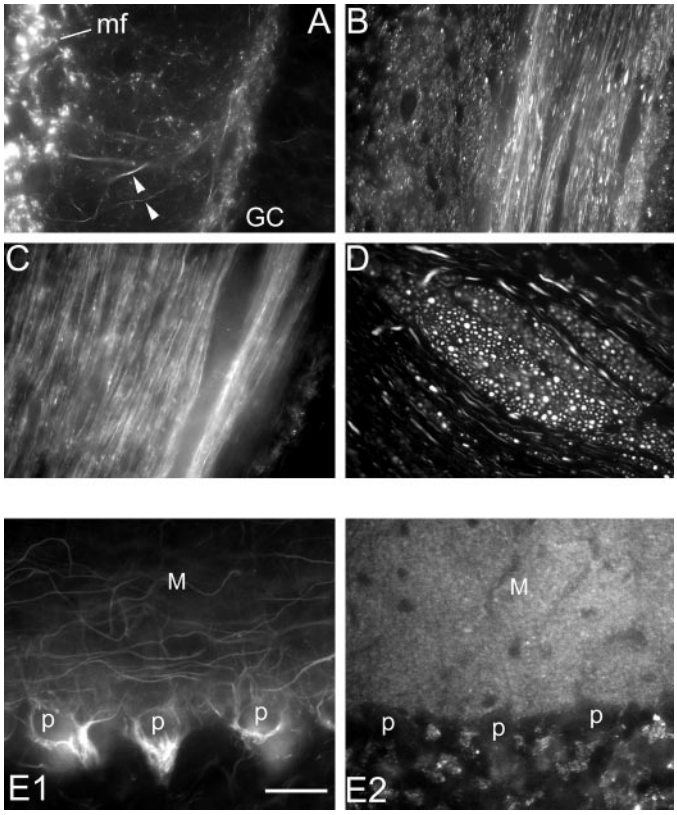

Figure 5. Axonal localization of Syn-3C immunoreactivity in adult rat brain, revealed by immunofluorescence microscopy. Localization of syndecan-3 in axons is shown in the dentate gyrus of the hippocampus (*A*), the corpus callosum (*B*), the stria terminalis (*C*), the white matter of the cerebellum (an axon bundle is cut in cross section) (*D*), and basket cells of the cerebellum (*E*). In *E1* and *E2*, the same field is double labeled by Syn-3C (*E1*) and synaptophysin (*E2*) antibodies. *M*, Molecular layer of the cerebellum; *mf*, mossy fiber tract; *p*, Purkinje cell bodies. Scale bar, 40 μm.

Axonal distribution of CASK in developing rat brain

Because CASK is complexed with syndecan-3, at least in part (Fig. 3*B*), we examined whether CASK might also be localized in axons. In adult rat brain, CASK is distributed predominantly in a somatodendritic pattern, with enrichment at synaptic sites (Hsueh et al., 1998). However, in contrast to PSD-95, CASK is expressed throughout brain development, with protein levels at embryonic stages (E14 and E18) as high, if not higher, as in adult brain (Fig. 6*A*). Immunohistochemistry was performed to determine CASK localization from P3 to P42. At P3, CASK immunoreactivity is indeed prominently found in axon tracts and white matter, such as the corpus callosum, mossy fiber tract, alveus, and fimbria of the hippocampus and the internal capsule and white matter of the cerebellum (Fig. 6*B,C*). In this respect, the distribution of CASK is remarkably similar to that of syndecan-3 in the first week or so after birth [compare Figs. 4*A* with 6*B* (P7)]; thus CASK is appropriately located to be interacting with syndecan-3 in axons of developing brain. In addition to the axonal staining, however, CASK immunoreactivity is also present in a somatodendritic pattern, even in early postnatal brain. With increasing age, CASK staining in the forebrain and cerebellum shifts progressively from a predominantly axonal pattern to a predominantly somatodendritic pattern (Fig. 6*B,C*) (Hsueh et al., 1998). By the end of the third week, CASK is barely detectable in white matter but found mostly in a somatodendritic distribution, e.g., in Purkinje cells of the cerebellum (Fig. 6*C*) and in pyramidal cells of the forebrain (Hsueh et al., 1998) (data not shown). As noted above, the levels of syndecan-3 in axons also fall after the first 2 weeks of postnatal development.

Axonal distribution of syndecan-3 and CASK in cultured cortical neurons

To confirm further the axonal distribution of syndecan-3 and CASK in neurons, we performed immunofluorescence staining in primary cortical cultures. In double-labeling experiments, Syn-3C antibodies stained long, fine processes that were positive for Tau but negative for MAP2, indicating the specific localization of syndecan-3 in axons (Fig. 7*Aa,Ab*). Similar results were obtained for cultured hippocampal neurons (data not shown). Preincubation with the immunogen peptide for Syn-3C blocked Syn-3C staining of axons (Fig. 7*Ac*), indicating the specificity of the signal. CASK antibodies also stained axons intensely, and by double labeling, CASK could be shown to colocalize with syndecan-3 in the same axons (Fig. 7*Ad*). In addition, CASK was also present in dendritic processes that were negative for syndecan-3 (Fig. 7*Ad*). Thus, the immunocytochemistry of cultured neurons is consistent with findings in the brain; syndecan-3 is concentrated in axons, whereas CASK is both dendritic and axonal. The overlapping distribution of CASK and syndecan-3 in axons and their coimmunoprecipitation from brain extracts are consistent with the direct association of CASK and syndecan-3 in the axonal compartment.

Differential subcellular localization of syndecan-2 and -3

Syndecan-2 is localized in central neuronal synapses (Hsueh et al., 1998), where it is highly colocalized with synaptophysin (Fig. 7*B*). By contrast, the pattern of labeling of syndecan-3 shows no overlap with synaptophysin staining, even in neuropil regions of the brain (Fig. 7*B*). Thus syndecan-2 (in synapses) and syndecan-3 (in axons) are segregated in complementary compartments; syndecan-3 seems to be mainly axonal in distribution, with little encroachment into axon terminals.

The Syn-2C antibodies cross-react with syndecan-4, which is expressed in glial cells *in vivo* (see Figs. 1, 2). The synaptic Syn-2C immunoreactivity is unlikely to arise from glial cells, however, because our previous immunogold EM studies have shown the Syn-2C labeling to be specifically associated with the postsynaptic density and the presynaptic terminal at brain synapses (Hsueh et al., 1998). Moreover, we have not observed glial staining in the brain with our Syn-2C antibodies, suggesting that their cross-reactivity *in vivo* is not significant.

Developmental pattern of syndecan-2 expression

HSPGs bind to extracellular matrix proteins and signaling factors; thus changes in HSPG expression and distribution could play a role in neuronal migration, neurite extension, and synapse formation during development of the nervous system. We found no obvious change in the axonal distribution of syndecan-3 during postnatal brain development, except for a sharp reduction in staining intensity after the first 2 weeks. Because syndecan-2 is enriched at synaptic junctions and may play a role in synaptogenesis, we were particularly interested in the developmental regulation of syndecan-2 distribution. We examined in detail the pattern of Syn-2C staining during the development of the cerebellum, which progresses through a well-characterized postnatal maturation in the rat. Syn-2C labeling during cerebellar synaptogenesis was compared with the distribution of synaptophysin (Fig.

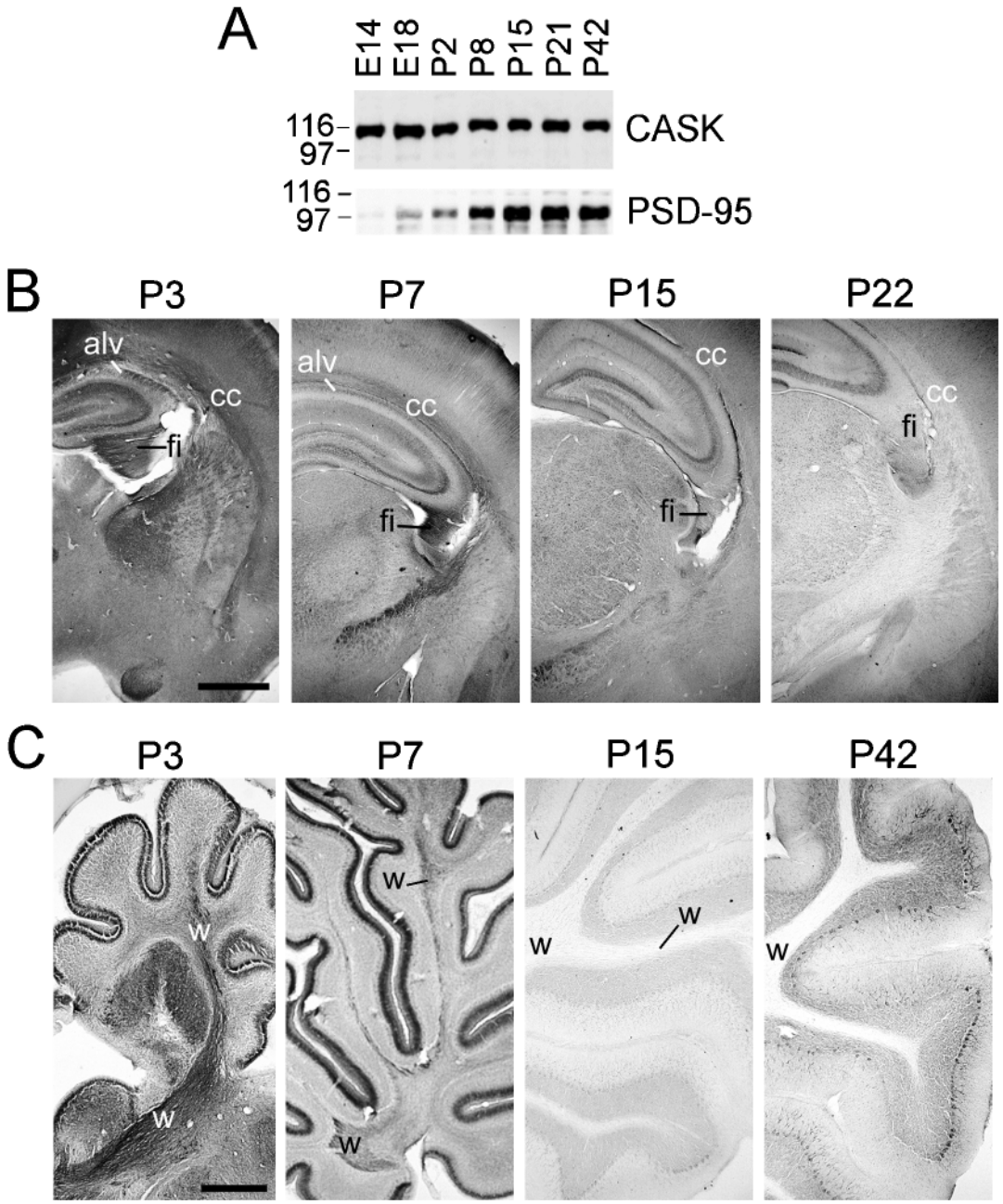

Figure 6. Expression and subcellular distribution of CASK during rat brain development. *A,* Developmental expression of CASK in rat brain. Ten micrograms of brain membrane fractions from rats at the indicated ages were immunoblotted with CASK and PSD-95 antibodies. *B*, *C,* Immunohistochemistry for CASK in forebrain (*B*) and cerebellum (*C*) at the indicated postnatal ages. Scale bars: *A,* 1 mm; *B,* 0.5 mm.

8*A*). In addition, we used anti-calbindin-D antibody (which strongly labels Purkinje cells and their dendrites) to delineate Purkinje cells and the molecular layer of the developing cerebellar cortex (Fig. 8*B*).

From as early as P3 through adulthood, the layer-specific pattern of Syn-2C staining matches that of synaptophysin (Fig. 8*A*). At P3, coexpression of syndecan-2 and synaptophysin occurs in the developing Purkinje cell layer (P) and in the internal granular layer (IGL). In contrast, both syndecan-2 and synaptophysin are absent from the external granular layer (EGL), where the granule cells are immature. Expression of syndecan-2 and synaptophysin increases in parallel, such that at P7 and P15, there is very intense labeling in the developing molecular layer (ML), with notable sparing of Purkinje cell bodies. During these first 2 weeks, syndecan-2 and synaptophysin also become increasingly expressed in the IGL, where the distributions of both proteins gradually condense into glomerular structures by P22 (Fig. 8*A*). The correlated distribution and intensity of Syn-2C and synaptophysin staining suggest that syndecan-2 is being selectively expressed in those areas of the developing cerebellum that are undergoing active synaptogenesis, namely, in the molecular layer, where granule cell parallel fiber axons are synapsing with Purkinje cell dendrites, and in the IGL, where granule cell dendrites form glomerular synapses with ascending mossy fibers. In the

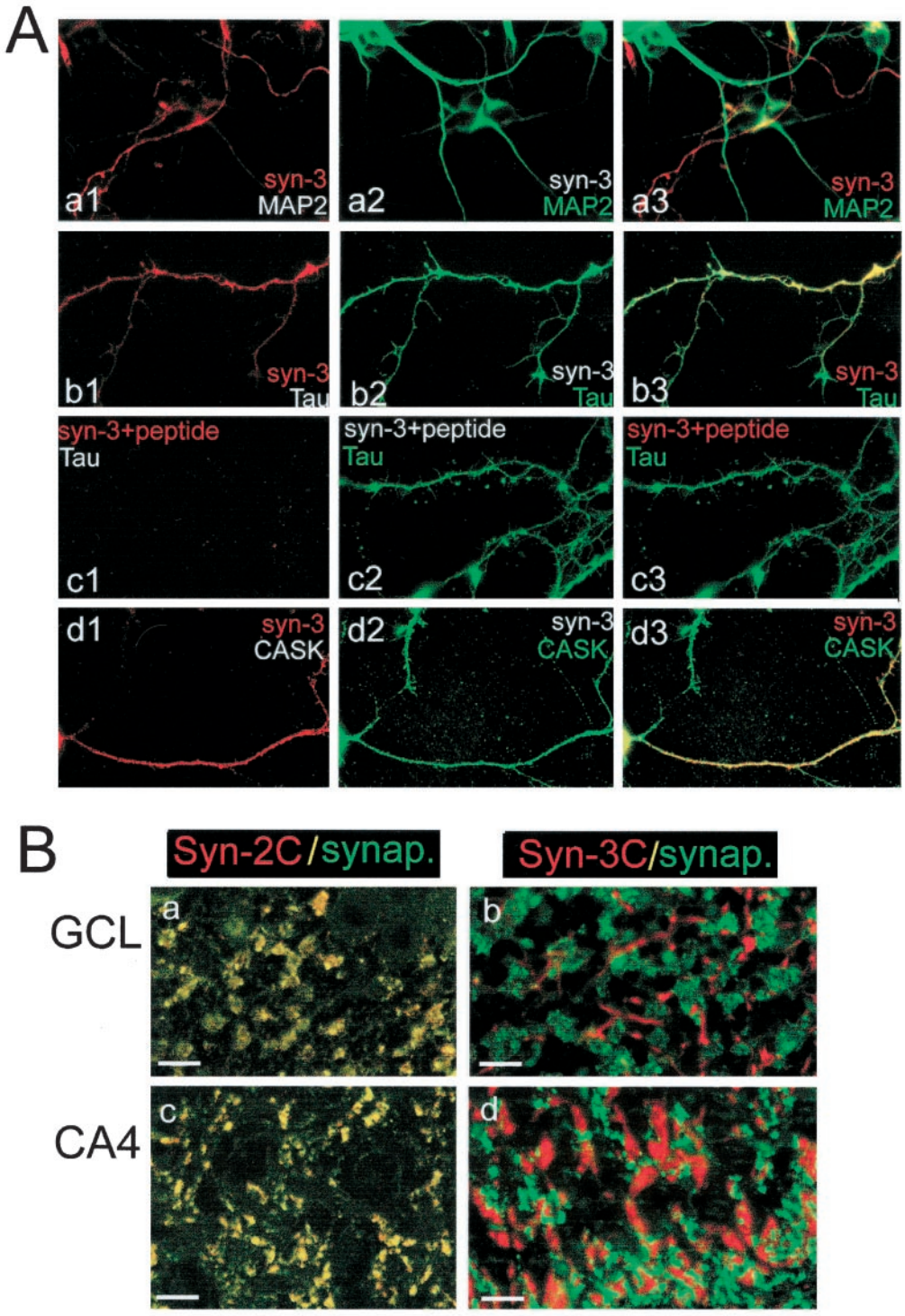

Figure 7. Syndecan-3 is localized in axons but excluded from synapses. *A,* Axonal localization of syndecan-3 in cultured cortical neurons. Double immunofluorescence labeling of cultured cortical neurons with Syn-3C and MAP2 (*a*), Syn-3C and Tau (*b*, *c*), and Syn-3C and CASK monoclonal K56A/50 (*d*) is shown. In *c*, Syn-3C antibodies were preincubated with syndecan-3 antigenic peptide. Each *set* of *images* (*a1–a3*, *b1–b3*, etc.) represents the same field visualized for syndecan-3 (Cy3, *red; left*) or for MAP2, Tau, or CASK (FITC, *green; middle*). The *right image* is an overlay of the first two (showing colocalization in *yellow*). *B,* Subcellular segregation of syndecan-2 and -3 in adult rat brain, revealed by double immunostaining with synaptophysin. Sections were double labeled with Syn-2C and synaptophysin antibodies (*synap.*) (*a, c*) or Syn-3C and synaptophysin antibodies (*b, d*). Confocal *images* were collected from the granule cell layer (*GCL*) of the cerebellum (*a*, *b*) and region CA4 of the hippocampus (*c*, *d*). Syndecan-2 and -3 were visualized by Cy3 (*red*), and synaptophysin was visualized by FITC (*green*) secondary antibodies. Scale bars, 20 μm.

mature cerebellum (P22), the intensity of Syn-2C staining falls, particularly in the deeper half of the molecular layer. At this stage, the glomeruli of the granule cell layer are the most prominent sites of Syn-2C as well as synaptophysin staining. In summary, the temporal and spatial pattern of syndecan-2 distribution in the developing cerebellum broadly matches that of the synaptic marker synaptophysin, indicating that expression of syndecan-2 occurs in step with synaptogenesis.

Interestingly, high-resolution confocal imaging reveals that syndecan-2 does not colocalize with synaptophysin at early developmental time points. Syn-2C immunoreactivity is highly punctate in nature as early as P3 in the cerebellum, as is synaptophysin (Fig. 9, *left*). However, at P3, the syndecan-2 puncta were less abundant than synaptophysin hot spots and showed little overlap with them. During the next 2 weeks of postnatal development, however, the degree of colocalization of syndecan-2 and synaptophysin increased. By P22, the distribution of syndecan-2 almost exactly matched the distribution of synaptophysin-positive synapses (Fig. 9, *left*). Similar results were obtained in region CA3 of the hippocampus. At P3 and P7, Syn-2C immunoreactivity is

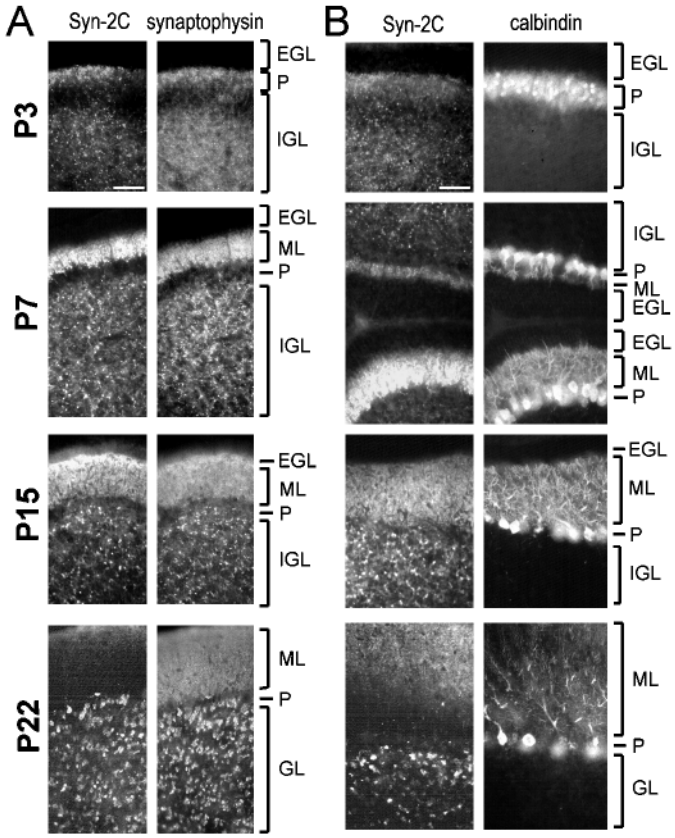

Figure 8. Coordinate layer-specific expression of syndecan-2 and synaptophysin in developing rat cerebellum. Double-label immunofluorescence of the cerebellum at P3, P7, P15, and P22 with Syn-2C and synaptophysin antibodies (*A*) or with Syn-2C and calbindin antibodies (*B*). Synaptophysin and calbindin were used as markers for synapses and Purkinje cells, respectively. Each *pair* of *images* represents confocal *images* of the same field visualized for one or the other antibody, as indicated. Scale bars, 100 μm.

present in cell bodies of the pyramidal cell layer and in a punctate pattern in the stratum lucidum; these puncta colocalized poorly with synaptophysin (Fig. 9, *right*). With maturation, the level of syndecan-2 staining in pyramidal cell bodies decreased, whereas punctate syndecan-2 colocalization with synaptophysin increased in the stratum lucidum. Collectively, these data indicate that the accumulation of syndecan-2 in synapses is a relatively late event in synapse assembly, occurring after clustering of synaptophysin.

DISCUSSION

Differential subcellular distribution of syndecan-2 and -3 in neurons

In this study we have shown that each member of the syndecan family of cell surface HSPGs exhibits a distinctive cell type-specific expression pattern in rat brain. Syndecan-1 is primarily restricted to cerebellar granule cells, whereas syndecan-4 is specifically expressed in glial cells. Recently, Ethell and Yamaguchi (1999) have found by immunostaining that syndecan-4 is specifically expressed by astrocytes in hippocampal culture, in agreement with our ISH findings *in vivo*. In forebrain neurons, syndecan-2 and -3 (the latter known previously as neuronal- or N-syndecan) are predominant. The functional significance of these cell type-specific expression patterns is unknown.

Even though their mRNAs are expressed within overlapping populations of neurons, syndecan-2 and -3 are segregated at the

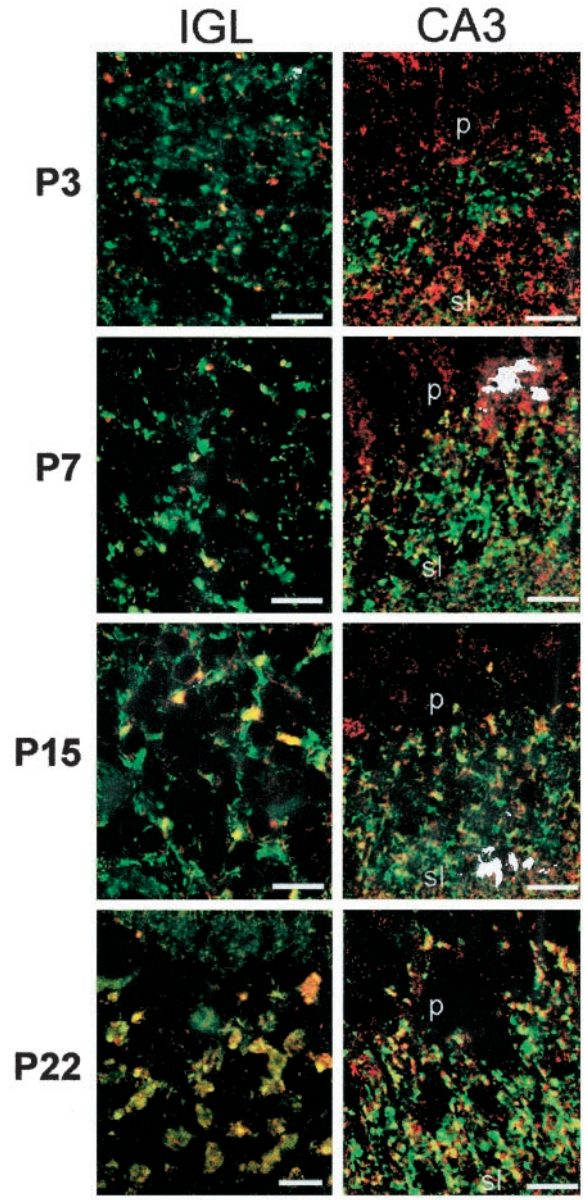

Figure 9. Synaptic localization of syndecan-2 occurs late in synapse development. Relationship of Syn-2C and synaptophysin staining in the *IGL* of the cerebellum and in the *CA3* region of the hippocampus during postnatal development examined by double-label immunofluorescence confocal microscopy at P3, P7, P15, and P22. Syn-2C is visualized by Cy3 (*red*), and synaptophysin is visualized by FITC (*green*). The *composite images* show colocalization in *yellow*. *P*, Pyramidal cell layer; *sl*, stratum lucidum. Scale bars: *IGL,* 10 μm; *CA3,* 20 μm.

protein level within neurons. Syndecan-2 is specifically localized to synapses (see also Hsueh et al., 1998), whereas syndecan-3 is concentrated in axons. What might be the mechanism underlying the differential subcellular distribution of these two neuronal syndecans? Targeting determinants for syndecan-2 and -3 may exist in the extracellular or intracellular domains of these transmembrane proteins. The extracellular regions of all syndecans show limited sequence similarity except at sites of GAG chain addition. This weak conservation of primary structure has conventionally been interpreted as reflecting the fact that it is the HS side chains rather than the core protein that mediate the important extracellular interactions of syndecans. However, it remains

possible that targeting signals are contained in the divergent extracellular regions. By contrast, there is high sequence conservation in the short cytoplasmic C-terminal tails of syndecans (see Fig. 2*A*) (for review, see Bernfield et al., 1992; David, 1993; Carey, 1997; Rapraeger and Ott, 1998). The C-terminal sequence -EFYA has been shown to bind to the PDZ domains of syntenin (Grootjans et al., 1997) and CASK (Cohen et al., 1998; Hsueh et al., 1998). Because all syndecans share an identical -EFYA C-terminal sequence, it seems unlikely that their subcellular segregation can be primarily dictated by differential interactions with CASK, syntenin, or other PDZ domain proteins. Indeed, the -EFYA C-terminal motif is not required for the localization of syndecan-2 in dendritic spines (Ethell and Yamaguchi, 1999). Outside of the C terminal and the juxtamembrane region, however, significant sequence differences between syndecans occur in the middle of the cytoplasmic tail. This region of syndecan-4 is reported to bind specifically to protein kinase C (Oh et al., 1997). It is possible that the "middle" cytoplasmic domain of syndecans can associate with different intracellular proteins involved in targeting and/or signaling. Domain swap experiments between syndecan-2 and -3 would be a reasonable approach to sort out the mechanisms for the differential targeting of these proteins. More challenging will be figuring out why different syndecans should be specifically sorted to distinct regions of the neuron.

Proteolytic cleavage of syndecan

We obtained direct evidence of extracellular cleavage of syndecan-3 *in vivo* by the loss of linkage between the extracellular Syn-3ecto epitope and the cytoplasmic Syn-3C epitope. The great majority of syndecan-3 appears to lack the cytoplasmic tail, on the basis of semiquantitative immunoprecipitation results (Fig. 2*D*). Consistent with this idea is that syndecan-3 purified from brain fails to react with an antibody directed against the cytoplasmic domain (Bernfield et al., 1993).

The cleavage and shedding of the majority of syndecan ectodomains *in vivo* change the functional implications of syndecan action. Syndecans are generally considered cell surface HSPGs involved in cell–matrix adhesion or low-affinity coreceptors for heparin-binding growth and/or differentiation factors. After cleavage, however, syndecan ectodomains can be shed from the plasma membrane and associate with the extracellular matrix rather than the cell surface. In this way, syndecan ectodomains may provide HSPGs to the ECM, perhaps contributing to an extracellular HSPG reservoir for retention and storage of heparin-binding factors (Loeb and Fischbach, 1995; Loeb et al., 1999). It is also plausible that syndecan ectodomains are coreleased with secreted molecules that bind to heparan sulfate, thereby acting as "chaperones" conveying heparin-binding factors into the extracellular space. We note, for instance, that syndecan-2 is present in presynaptic terminals as well as at postsynaptic sites (Hsueh et al., 1998) and that it may be part of an exocytosis complex via its interaction with CASK (Butz et al., 1998). It will be interesting therefore to determine whether the degree of "shedding" is similar for all the syndecan family members and how this process is regulated during development or in response to neural activity.

Changing distribution of CASK during brain development and its axonal colocalization with syndecan-3

We showed previously that CASK is distributed in a somatodendritic pattern in adult brain and that it is coenriched with syndecan-2 at synaptic junctions (Hsueh et al., 1998). Here we report that the subcellular distribution of CASK is prominently axonal in early postnatal brain. In its developmental shift from an axonal to a synaptic distribution, CASK resembles several other proteins such as Fasciclin II (Zito et al., 1997); Discs large (Lahey et al., 1994), and Eph receptor tyrosine kinases (Torres et al., 1998). The functional significance of syndecan-3–CASK interactions in developing axons remains to be determined. On the basis of the binding properties of cell surface HSPGs, we speculate that the syndecan-3–CASK complex may be involved in adhesive or signaling functions during axon growth, migration, and/or fasciculation. In this context, it is noteworthy that syndecan-3 has been isolated as a receptor or coreceptor for the heparin-binding growth-associated molecule, a molecule that promotes neurite outgrowth (Raulo et al., 1994). Syndecan-3 has also been reported to copurify with cortactin and Fyn tyrosine kinase and to show increased expression in response to synaptic activity (Lauri et al., 1999).

Neurexins, a highly heterogeneous family of neuronal cell surface proteins, are also binding partners for the PDZ domains of CASK (Hata et al., 1996; Missler et al., 1998). Like that of the syndecans, neurexin immunoreactivity has been localized at synapses (Ushkaryov et al., 1992) and on axons (Russell and Carlson, 1997). It will be interesting to determine how the expression level and subcellular distribution of neurexins are regulated during development.

Role of syndecan-2 in synapses

Although syndecan-2 is specifically localized in synapses in adult brain, our data suggest that its concentration in synapses (as defined by synaptophysin clusters) occurs relatively late in synapse development. Thus synaptic contacts form initially in the absence of detectable syndecan-2. Our results *in vivo* are consistent with the findings of Ethell and Yamaguchi (1999) *in vitro.* These investigators found that syndecan-2 was localized specifically in dendritic spines of cultured hippocampal neurons but that expression of syndecan-2 was detectable only after 2 weeks *in vitro* and increased in the following weeks. This time course lags behind that of synapse formation in hippocampal culture (Rao et al., 1998). Moreover, forced expression of syndecan-2 in neurons at an early stage in culture (when endogenous syndecan-2 is not normally expressed) accelerated the development of dendritic spine morphology but did not affect the number of spines or synapses (Ethell and Yamaguchi, 1999). Taken together with the late accumulation of syndecan-2 in synapses, these findings imply that syndecan-2 is involved in a late stage of synaptic development, such as the morphological maturation of dendritic spines, rather than in specifying the formation of synapses.

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