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Cerebroglycan, a Developmentally Regulated Cell-Surface Heparan Sulfate Proteoglycan, Is Expressed on Developing Axons and Growth Cones

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Cerebroglycan is a glycosylphosphatidylinositol-linked integral membrane heparan sulfate proteoglycan found exclusively in the developing nervous system. In the rodent, cerebroglycan mRNA first appears in regions containing newly generated neurons and typically disappears 1 to several days later (Stipp *et al.*, 1994, *J. Cell Biol.* 124:149–160). To gain insight into the roles that cerebroglycan plays in the developing nervous system, monospecific antibodies were prepared and used to localize cerebroglycan protein. In the rat, cerebroglycan was prominantly expressed on axon tracts throughout the developing brain and spinal cord, where it was found at times when axons are actively growing, but generally not after axons have reached their targets. Cerebroglycan was also found on neuronal growth cones both *in vivo* and *in vitro*. Interestingly, cerebroglycan immunoreactivity was rarely seen in or around neuronal cell bodies. Indeed, by examining the hippocampus at a late stage in development—when most neurons no longer express cerebroglycan but newly generated granule neurons do—evidence was obtained that cerebroglycan is strongly polarized to the axonal, and excluded from the somatodendritic, compartment of neurons. The timing and pattern of cerebroglycan expression are consistent with a role for this cell-surface heparan sulfate proteoglycan in regulating the growth or guidance of axons.

INTRODUCTION

Heparan sulfate proteoglycans (HSPGs) have been hypothesized to play important roles in nervous system development (Lander, 1993) and have been implicated in such diverse processes as cell–cell adhesion (Reyes *et al.*, 1990; Stanley *et al.*, 1995), cell-substratum adhesion (LeBaron *et al.*, 1988; Haugen *et al.*, 1992), neurite outgrowth (Wang and Denburg, 1992), and growth factor signaling (Yayon *et al.*, 1991; Kan *et al.*, 1993; Aviezer and Yayon, 1994; Zioncheck *et al.*, 1995).

Although several distinct cell surface HSPGs are expressed in the developing nervous system (Herndon and Lander, 1990; Lander, 1993), little is known about their physiological functions. Molecular cloning suggests that the core proteins of most cell surface HSPGs fall into two families: the syndecan family of transmembrane HSPGs and the glypican family of glycosylphosphatidylinositol (GPI)-anchored HSPGs. To date, four syndecan family members have been described (Bernfield *et al.*, 1992). One member of the syndecan family, syndecan-3, is highly expressed in the developing brain (Carey *et al.*, 1992; Gould *et al.*, 1995).

The glypican family is currently composed of four mammalian proteins: glypican, cerebroglycan, OCI-5, and K-glypican, as well as the product of the *Drosophila dally* gene (Lander *et al.*, 1996). The amino acid sequences of these GPI-anchored HSPGs show considerable homology throughout their length, including an invariant pattern of 14 cysteines that confers upon these molecules a compact, disulfide-stabilized tertiary structure. Two of these HSPGs, cerebroglycan and glypican, have been directly isolated from developing rat brain (Karthikeyan *et al.*, 1992, 1994; Litwack *et al.*, 1994; Stipp *et al.*, 1994), and it appears that these, along with syndecan-3, are the major carriers of cell

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surface heparan sulfate in the developing brain. By *in situ* hybridization, cerebroglycan expression is restricted to the developing nervous system where it is transiently expressed in regions containing newly postmitotic neurons (Stipp *et al.*, 1994). In contrast, glypican mRNA is expressed in ventricular zones in the developing rat brain and by many projection neurons throughout development and into adult life (Karthikeyan *et al.*, 1992, 1994; Litwack *et al.*, 1994). The mRNAs encoding K-glypican and OCI-5 have also been detected at low levels in the developing and adult rodent brain (Watanabe *et al.*, 1995), and although their expression patterns have not been thoroughly characterized to date, K-glypican mRNA has been detected in the ventricular zone of the developing cerebral cortex (Watanabe *et al.*, 1995). The distribution of OCI-5 is currently unknown.

To investigate the roles that HSPGs of the glypican family play in neuronal growth and development, we have raised specific polyclonal antibodies to the core protein of cerebroglycan and used these antibodies to localize cerebroglycan during the development of the rodent central nervous system. As described below, cerebroglycan is localized primarily, if not exclusively, to the growing axons and growth cones of newly generated neurons, a result that suggests a function for this HSPG in axonal growth and/or guidance.

METHODS

Antipeptide Antibodies

The peptide CRPPRPPRPPRRDGL, which corresponds to amino acids 521–535 of rat cerebroglycan (Stipp *et al.*, 1994), was synthesized and purified by reverse-phase HPLC (Biopolymers Lab, M.I.T.) and coupled to keyhole limpet hemocyanin (KLH) as follows: 20 mg of KLH (Pierce Chemical Co., Rockford, IL) was activated by reaction with 2 mg sulfo-SMCC (Pierce) at room temperature with stirring for 30 min. Activated KLH was purified over a Sephadex G-25 column (Pharmacia) equilibrated in 0.1 *M* sodium phosphate (pH 6.0). Twenty-two milligrams of peptide 521 was dissolved in the same buffer and allowed to react overnight at room temperature with the activated KLH. KLH-521 complexes were purified over a Sephadex G-25 column. Rabbits were injected intradermally with 2.5 mg KLH-521 in complete Freund's adjuvant and boosted twice intramuscularly with 2.5 mg KLH-521 in incomplete Freund's adjuvant. Antisera were collected and subsequently affinity-purified over peptide 521 coupled to Sulfo-Link (Pierce); the resulting affinity-purified antibody was designated 521-2. An anti-glypican antibody, designated 343-1 and directed against amino acids 343–360 of rat glypican, was prepared in a similar fashion (E. D. Litwack *et al.*, in preparation).

Immunohistochemistry

Embryonic Sprague–Dawley rats were removed from timed pregnant females and fixed by overnight immersion in 4% paraformaldehyde in Ca^{2+}/Mg^{2+}-free Dulbecco's phosphate-buffered saline (PBS) at 4°C and subsequently cryoprotected in 15% sucrose in PBS. Date of sperm positivity was considered to be Embryonic Day 1 (E1). Brains from postnatal animals were quickly removed and quick-frozen in isopentane on dry ice. Twenty-micrometer cryostat sections of all samples were collected on Probe-On Plus slides (Fisher) and stored at −80°C. Unfixed tissue sections were postfixed by immersion in 4% paraformaldehyde in PBS for 10 min at room temperature prior to storage.

For immunohistochemistry, sections were incubated in blocking solution (2% bovine serum albumin [BSA]; 3% goat serum; 100 m*M* Tris–Cl, pH 7.5; 150 m*M* NaCl; 0.3% Triton X-100). Sections were washed in TBS (100 m*M* Tris, pH 7.5; 150 m*M* NaCl) and, if sections were to be developed with horseradish peroxidase, treated twice for 30 min each in 0.3% H_2O_2 in methanol and washed again in TBS. Affinity-purified 521-2 antibody, diluted in blocking solution, was applied overnight at room temperature at a concentration of 2.5–5 μg/ml. For immunofluorescence, a Cy3-conjugated goat anti-rabbit antibody (Jackson Immunoresearch) was used at a dilution of 1:100 in blocking solution as a secondary antibody. Sections were coverslipped with a solution of ProLong antifade reagent (Molecular Probes) in GelMount (Biomeda). For immunoperoxidase staining, a biotin-conjugated goat anti-rabbit antibody (Vector) diluted 1:300 in blocking solution was used as a secondary antibody. Sections were washed in TBS and then incubated in ABC reagent (Vector) diluted in 0.1 *M* Tris (pH 7.5). Sections were washed again in 0.1 *M* Tris and developed in 50 μg/ml DAB/0.3% H_2O_2/0.1 *M* Tris. Sections were washed in H_2O, dehydrated, cleared in xylenes, and coverslipped using Permount. In some cases, sections were counterstained with either methyl green (0.1%) or bisbenzamide (20 μg/ml) prior to dehydration and mounting. Nonspecific staining was defined as staining that occurred in the absence of primary antiserum. Sections were examined on a Zeiss Axiophot microscope equipped for brightfield, DIC, and epifluorescence.

Proteoglycan Isolation and Radioiodination

Proteoglycans (PGs) were isolated from detergent extracts of neonatal rat brain membranes by sequential salt elution from DEAE-Sephacel (Pharmacia) essentially as described (Herndon and Lander, 1990). For radioiodination, PGs were rebound to approximately 50 μl of DEAE-Spectra/Gel (Spectrum) in 3–4 volumes of 0.05 *M* Tris, pH 8.0, containing 0.5% CHAPS overnight at 4°C. The beads were isolated, washed with 0.05 *M* Tris, pH 8.0, to remove detergent, and transferred to a polypropylene tube with a minimum volume of buffer. PGs were iodinated with 5 mCi of $Na^{125}I$ (NEN Dupont). Iodinations were initiated with the addition of 4 μl of a 1 mg/ml solution of chloramine T, allowed to proceed for 3 min at room temperature, and terminated with the addition of 43 μl each of 10 m*M* sodium metabisulfite and 10 m*M* KI. Beads were transferred to a column, washed with 20 ml of 0.05 *M* Tris, pH 8.0; 0.5% CHAPS; 150 m*M* NaCl and eluted with 8 × 50-μl aliquots of 0.05 *M* Tris, pH 8.0; 0.5% CHAPS; 750 m*M* NaCl. Fractions containing the peak radioactivity were pooled and brought to 1 mg/ml BSA. Aliquots were flash frozen on dry ice/ethanol and stored at −80°C. Protein determinations were made prior to the addition of BSA using the amido black method essentially as described (Herndon and Lander, 1990) with crystalline BSA as standard, except that proteins were precipitated with 10% trichloroacetic acid (TCA).

Glycosaminoglycan (GAG) Lyase Digestion, SDS–PAGE, and Western Blotting

Digestions with heparitinase, chondroitinase ABC (Seikagaku), or both enzymes were performed on ^{125}I-labeled PGs as described

(Herndon and Lander, 1990), in the presence of 1 m*M* EDTA, 1 μg/ml phenylmethylsulfonyl fluoride (PMSF), 25 μg/ml *N*-ethyl maleiimide (NEM), and 1 μg/ml pepstatin A, in a volume of 30 μl. All incubations were carried out at 37°C except digestions with heparitinase alone, which were performed at 43°C to inhibit any contaminating chondroitinase activity. After 4–6 hr, digestions were terminated by the addition of 7.5 μl 5× SDS sample buffer, heated to 95°C for 10 min, and analyzed by SDS–PAGE. For Western blotting, neonatal brain membranes were subjected to GAG lyase digestion in the presence of protease inhibitors, resolved by SDS–PAGE, and electrophoretically transferred to Immobilon P. To block nonspecific protein binding, the membrane was incubated in TBS containing 3% goat serum, 2% BSA, and 0.3% Tween 20 for 30–60 min, followed by incubation overnight in the same buffer containing 2.5 μg/ml 521-2 antibody. Incubation with enhanced chemiluminescence reagents was performed according to the instructions of the manufacturer (Amersham, Arlington Heights, IL).

Immunoprecipitation

Immunoprecipitations were performed from ^{125}I-labeled PGs starting with 0.5×10^6 TCA-precipitable cpm of material. PGs were subjected to digestion with appropriate GAG lyases, brought to a volume of 200 μl with the addition of 0.05 *M* Tris, pH 8.0; 0.5% CHAPS; 150 m*M* NaCl (buffer C) containing EDTA and PMSF, and cleared by the addition of 30 μl of a 50 mg/ml solution of protein A–Sepharose beads (Sigma) in the same buffer. After rocking for 30–60 min at 4°C, beads were pelleted by centrifugation and the supernatants transferred to fresh tubes. Antibodies were added and the tubes were incubated overnight at 4°C with rocking. Immune complexes were collected on protein A–Sepharose for several hours at 4°C by the addition of 50 μl protein A–Sepharose. Beads were collected by centrifugation, washed once with 1 ml buffer C, once with buffer C containing 750 m*M* NaCl, and again with buffer C. Beads were eluted with 30 μl 2× SDS sample buffer at 95°C for 10 min and the eluate was analyzed by SDS–PAGE. In some experiments, samples were washed before elution with 50 m*M* Tris, pH 8.0; 150 m*M* NaCl; 1.0% SDS; 1 m*M* EDTA, 1 μg/ml PMSF; 25 μg/ml NEM; and 1 μg/ml pepstatin A. Identical results were obtained.

Growth Cone Particle Preparation

Growth cone particles (GCPs) were prepared from Postnatal Day 0 (P0) rat brains as described (Greenberger and Pfenninger, 1986; Skene *et al.*, 1986; Simkowitz *et al.*, 1989). Briefly, brains (including olfactory bulbs) were removed into ice-cold PBS, dissected free of meninges, and homogenized in 10 volumes GCP buffer (10 m*M* TES, pH 7.4; 1 m*M* $MgCl_2$; 0.32 *M* sucrose; PMSF (1 μg/ml); pepstatin A (1 μg/ml); and *N*-ethyl maleiimide (2.5 μg/ml). Homogenates were clarified by centrifugation at 5000*g* for 15 min at 4°C and gently layered onto discontinuous sucrose gradients consisting of 0.83 *M*, 1.0 *M*, and 2.0 *M* sucrose steps in GCP buffer. Gradients were centrifuged at 242,000*g* in a swinging bucket rotor at 4°C for 40 min. GCPs were isolated from a band of white material forming at the first sucrose interface.

Primary Cell Culture and Establishment of Ψ2 and PC12 Cell Lines Expressing Cerebroglycan

A cerebroglycan expression vector, pLNCX-CBGΔ3′, was constructed by subcloning the 1835-bp *Eag*I/*Nar*I restriction fragment containing the full open reading frame of the cerebroglycan cDNA (Stipp *et al.*, 1994) into the multicloning site of the pLNCX retroviral expression vector (Miller and Rosman, 1989). Ψ2 cells were transfected with pLNCX-CBGΔ3′ using lipofectamine (Gibco BRL) according to the instructions of the manufacturer. Conditioned medium from the transfected Ψ2 cells was used to infect Ψ2 cells treated with 200 ng/ml tunicamycin and 8 μg/ml polybrene, creating stable, virus-producing cell lines.

PC12 cells were grown in RPMI 1640 with 10% heat-inactivated horse serum, 5% fetal calf serum, glutamine, and penicillin/streptomycin on Vitrogen (Celltrix)-coated plates. Cells expressing cerebroglycan were generated by infecting PC12 cells with conditioned medium, containing 8 μg/ml polybrene, from Ψ2 producer cell lines. Infected PC12 cells were selected by growth in 1 mg/ml G418 and maintained as an uncloned population by growth in 0.5 mg/ml G418; the cells were designated PC12inf$_u$CBGΔ3′. For immunohistochemistry, PC12inf$_u$CBGΔ3′ cells were grown on laminin-coated coverslips (20 μg/ml). Cells were rinsed once in prewarmed RPMI and then fixed for 10 min at room temperature in 4% paraformaldehyde, 5% sucrose in PBS and processed for immunohistochemistry as described above using a Cy3-conjugated second antibody (Jackson Immunoresearch) at a dilution of 1:100. Coverslips were washed in TBS, mounted in Aquamount, and imaged on a confocal microscope (MRC-1024) using identical image acquisition parameters for both infected and parental control cell lines.

Embryonic Day 18 retinal neurons were cultured using timed pregnant rats as described (Calof *et al.*, 1994). Neurons were plated onto merosin (Life Technologies; 40 μg/ml)-coated glass coverslips. After 16–20 hr they were fixed and processed for immunofluorescence as described above.

RESULTS

Production and Characterization of Antipeptide Antibodies

A synthetic peptide corresponding to amino acids 521–535 of the cerebroglycan coding sequence was synthesized, coupled to KLH, and used to produce an antiserum in rabbits. The resulting serum, 521-2, was affinity-purified against the same peptide. To confirm the specificity of the affinity-purified antibody, 40 μg of neonatal rat brain membrane protein was digested with either heparitinase or chondroitinase or a mixture of both GAG lyases and subjected to Western blot analysis (Fig. 1). A single 52-kDa band was seen following heparitinase digestion, but not in untreated samples or samples subjected to chondroitinase digestion alone. In samples not treated with heparitinase (lanes C and U), a broad high-molecular-weight smear can faintly be seen, indicative of an undigested heparan sulfate proteoglycan. Digestion with both chondroitinase and heparitinase did not alter the mobility of the immunoreactive band further than was observed with heparitinase digestion alone, consistent with previous observations that cerebroglycan bears only heparan sulfate GAG chains (Herndon and Lander, 1990). The poor immunostaining of undigested cerebroglycan (lanes C and U) is most likely due to the poor binding of GAG-bearing PGs to protein blotting membranes (Rapraeger *et al.*, 1985) and not to an inability of the antise-

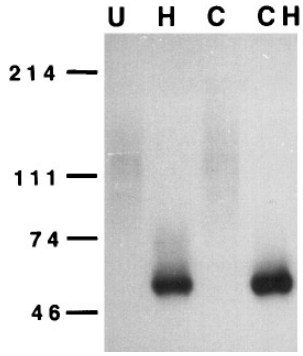


FIG. 1. Western blot of cerebroglycan in Postnatal Day 0 rat brain membranes. 18 μg protein was loaded in each lane of a nonreducing SDS gel, which was subsequently transferred to Immobilon P, probed with the 521-2 antibody, and processed for enhanced chemiluminescence. Lanes are marked as follows: U, untreated; H, heparitinase treated; C, chondroitinase treated; CH, heparitinase- and chondroitinase-treated. A single band of molecular weight 52 kDa is observed only following heparitinase digestion; chondroitinase treatment does not alter its migration further. In samples not treated with heparitinase (C and U), a broad high-molecular-weight smear can faintly be seen, indicative of an undigested heparan sulfate proteoglycan.

rum to recognize the intact cerebroglycan molecule, since 521-2 readily immunoprecipitates intact cerebroglycan from a complex mixture of proteoglycans (see below).

To prove the specificity of the antibody, PC12 cells, which do not express any endogenous cerebroglycan (M. E. Herndon, C. S. Stipp, and A. D. Lander, unpublished observations), were infected with a replication-defective retrovirus engineered to direct expression of a full-length cerebroglycan cDNA. Infected cells stained strongly with the 521-2 antibody (Fig. 2A) in a distinctly punctate pattern similar to that seen for other cell-surface GPI-anchored proteins (Sargiacomo *et al.*, 1993; Lisanti *et al.*, 1994). In contrast, the uninfected parental PC12 cell line showed only background levels of immunoreactivity (Fig. 2B).

Cerebroglycan Expression in the Developing Rat Brain

In situ hybridization has suggested that cerebroglycan mRNA is restricted to the developing nervous system and, furthermore, that the onset of cerebroglycan gene expression in different parts of the nervous system correlates with the terminal mitoses of neurons (Stipp *et al.*, 1994). In extracts of whole rat brain, cerebroglycan protein expression peaks near the day of birth (Herndon and Lander, 1990). To determine the localization of cerebroglycan in the developing nervous system, we immunostained cryostat sections of rat brain at several developmental ages, concentrating on P0. In parasaggital sections of P0 rat heads, cerebroglycan immunoreactivity was restricted to the nervous system (Fig. 3). Highest levels of immunoreactivity were seen in the cerebral cortex, anterior commissure, fornix, optic chiasm, deep layers of the cerebellum, superior and inferior colliculi, and midbrain and brain stem.

Close examination of these and other sections suggests that cerebroglycan is predominantly, if not exclusively, associated with developing axon tracts. For example, in saggital sections through the neocortex at P0 (Fig. 4A), high levels of cerebroglycan immunoreactivity are seen in the intermediate zone and in fibers that extend from the intermediate zone through the cortical plate toward the pial surface (arrows). This pattern of immunoreactivity corresponds to the pathway by which thalamic axons enter the cortex. These axons grow through the intermediate zone parallel to the ventricular surface before turning or sending collaterals that grow upward toward their termination zones in the cortical plate (Miller *et al.*, 1993). Although the same pathway is followed by descending cortical axons, at least some of the cerebroglycan immunoreactivity along this pathway is likely to be present on thalamocortical axons, since cerebroglycan mRNA is present at high levels in the thalamus at similar stages of development (Stipp *et al.*, 1994). Within the cortex, cerebroglycan-immunoreactivity is also seen in the marginal zone, a layer which consists primarily of intracortical axons. The ventricular zone, which contains proliferating neural precursors, but very few axons, is devoid of immunoreactivity. In control sections incubated in the absence of the primary antibody, no immunoreactivity is observed (Fig. 4B).

Cerebroglycan immunoreactivity can also be seen on fiber bundles coursing within the superficial layers of the superior colliculus at P0; these presumably correspond to the axons of retinal ganglion cells invading the stratum opticum (Fig. 4C), although afferents arising from the cerebral cortex also project to this region. In intermediate layers of the superior colliculus, fibers cut in cross section stain prominently. Based on their position and orientation, these fibers are likely to be tectobulbar axons (Fig. 4C) which run as tightly fasciculated bundles perpendicular to the ingrowing optic axons (Kroger and Walter, 1991). Cerebroglycan immunoreactivity can also be seen on presumptive corticotectal fibers in the deep layers of the superior colliculus (Fig. 4C). These are likely to correspond to inputs from cortical visual areas, but could also represent descending projections from the superior colliculus to the reticular formation, pontine nucleii, inferior olive, or spinal cord (Altman and Bayer, 1981b). However, since those descending fibers arise from cell bodies dispersed throughout all collicular layers, and little cerebroglycan mRNA has been observed in the superior colliculus at this age (Fig. 6I of Stipp *et al.*, 1994), the cerebroglycan-immunoreactive fibers most likely correspond to corticotectal afferents.

Cerebroglycan immunoreactivity is also seen in the hippocampal formation of the neonatal rat brain. In the coronal section shown in Fig. 4D, immunoreactivity is seen within the stratum radiatum, where both intrahippocampal commissural axons as well as the axons of the Schaffer collateral pathway from the hippocampal pyramidal neurons project.

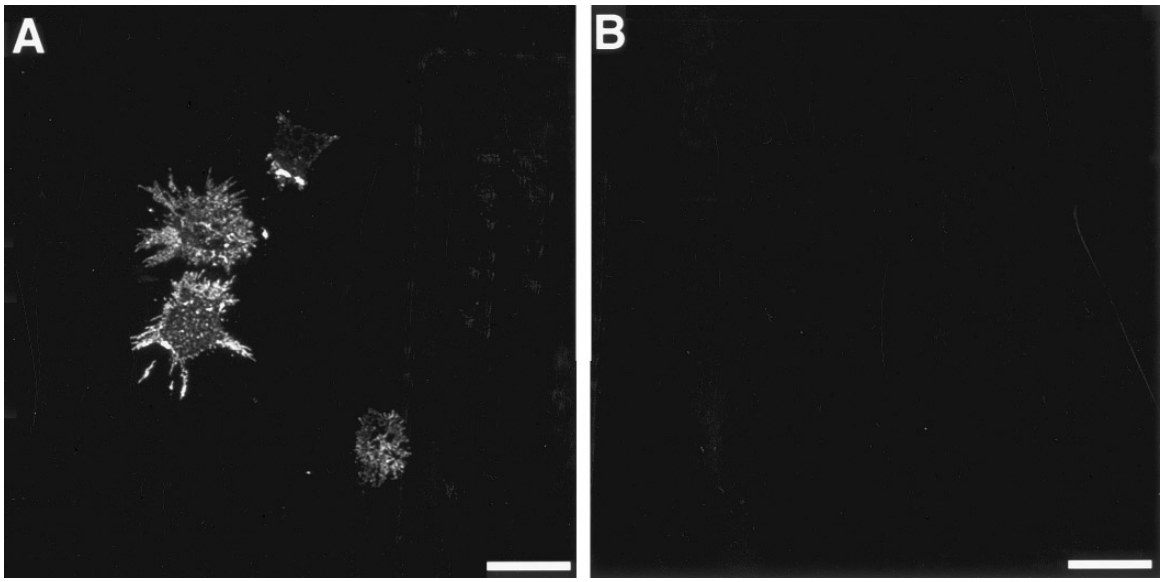


FIG. 2. Immunostaining of PC12 cells transfected with a cerebroglycan transgene. PC12 and PC12inf$_u$CBGΔ3′ cells were grown on laminin-coated coverslips and stained for cerebroglycan. Cerebroglycan immunoreactivity was visualized using a Cy3-conjugated second antibody. Coverslips were imaged on a MRC-1024 confocal microscope using identical image acquisition parameters for both PC12inf$_u$-CBGΔ3′ (A) and the parental PC12 cell line (B), demonstrating that cerebroglycan immunoreactivity is only seen in the transfected cell line. Bar, 15 μm.

Weak immunoreactivity is also seen in the mossy fiber layer, the site of axons that arise from granule neurons within the dentate gyrus and project toward area CA3. In the same section, prominent cerebroglycan immunoreactivity can be seen within several other axon pathways, including the optic tract, the fimbria of the fornix, the intermediate zone of the cerebral cortex, the corpus callosum, and the internal capsule (not shown). Other major axon tracts that stain strongly for cerebroglycan include the anterior commissure (Fig. 3), the posterior commissure (Fig. 4E), and spinal commissural axons (see below, Fig. 5).

The Timing of Cerebroglycan Expression Correlates with Stages of Cell Migration and Early Axon Growth

In many regions of the developing brain, regional variations in cerebroglycan immunoreactivity correlate with differences in the developmental stages of neurons. For example, in the inferior colliculus the highest levels of cerebroglycan immunoreactivity are seen in the most caudal and ventral regions (Fig. 4F), where the most recently postmitotic neurons are located (Altman and Bayer, 1981a). These are also the regions that hybridize most strongly for cerebroglycan mRNA at this stage (Stipp *et al.*, 1994). In the P0 cerebellum (Fig. 4G), the heaviest labeling is seen in the deep layers in a pattern that correlates with the migratory paths taken by neurons settling into the deep cerebellar nucleii (Altman and Bayer, 1985a). Weaker immunoreactivity can also be seen in a cell layer beneath the external granule layer, corresponding to the developing Purkinje cell layer. Immunoreactivity in this layer appears stronger in dorsal than in ventral cerebellum, a difference that correlates with the maturational gradient of Purkinje cell development. In dorsal regions of the rat cerebellum, Purkinje cells are still migrating into the Purkinje cell layer as late as Embryonic Day 22, but have completed their migrations several days earlier in the more posterior and ventral cerebellum (Altman and Bayer, 1985b). Thus, in the cerebellum as in the inferior colliculus, the strongest immunoreactivity is associated with the least mature postmitotic neurons.

In the olfactory bulb (Fig. 4H), weak cerebroglycan staining can be seen in the olfactory nerve layer, which contains the intermingled axons of olfactory receptor neurons at various stages of maturity (Shipley and Ennis, 1996). Intense cerebroglycan immunoreactivity is seen, however, in the rostral migratory stream of newly born interneurons which originate in the anterior subventricular zone and subsequently migrate into the olfactory bulb. Immunoreactivity is also apparent on the presumptive axons of mitral cells in the internal plexiform layer. In older animals, at P21, specific cerebroglycan immunoreactivity is still visible throughout the granular layer of the bulb, consistent with observations that granular neurons continue to be generated throughout the life of the rat (Kaplan and Hinds, 1977), as well as on axons within the lateral olfactory tract (not shown) which presumably correspond to the axons of mitral cells (see Discussion).

In the spinal cord, where neurogenesis is completed earlier

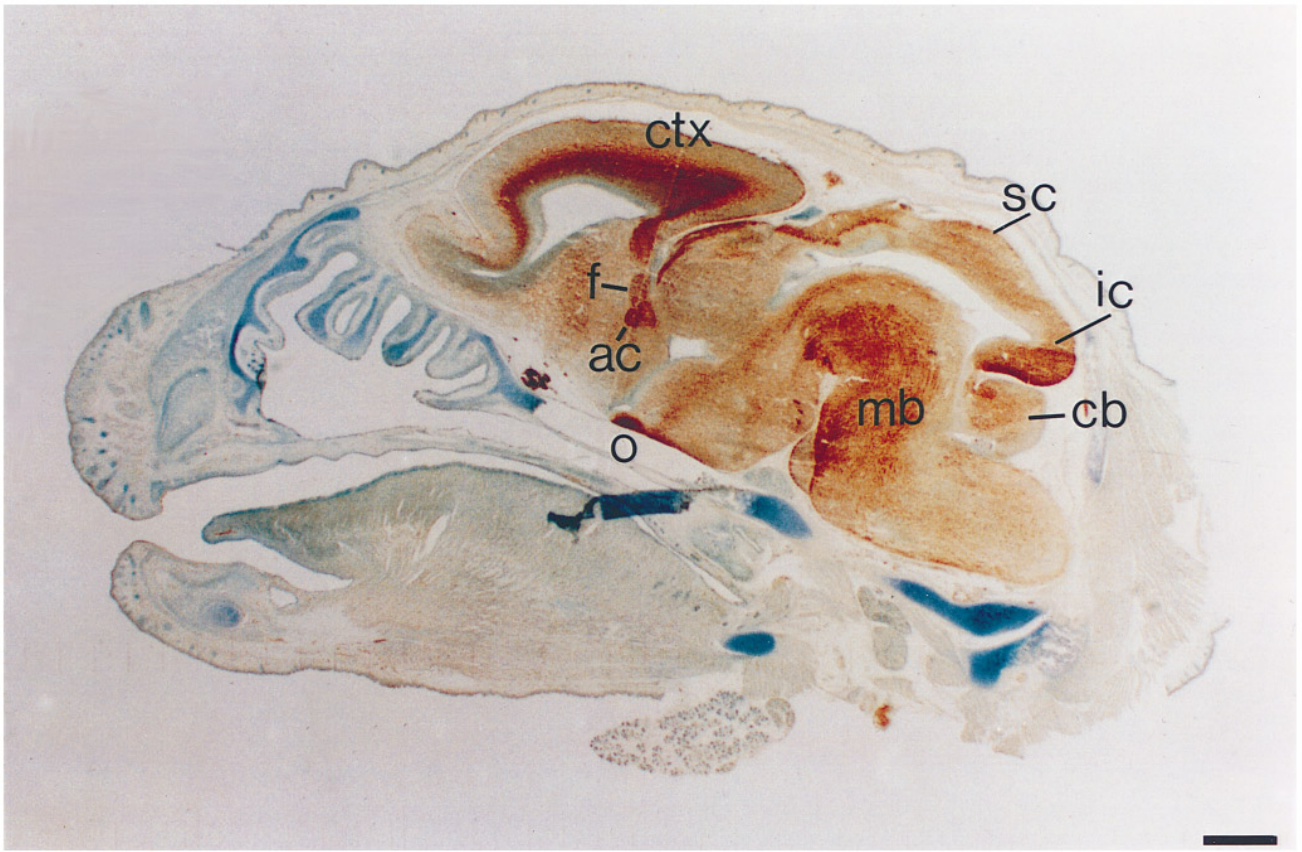


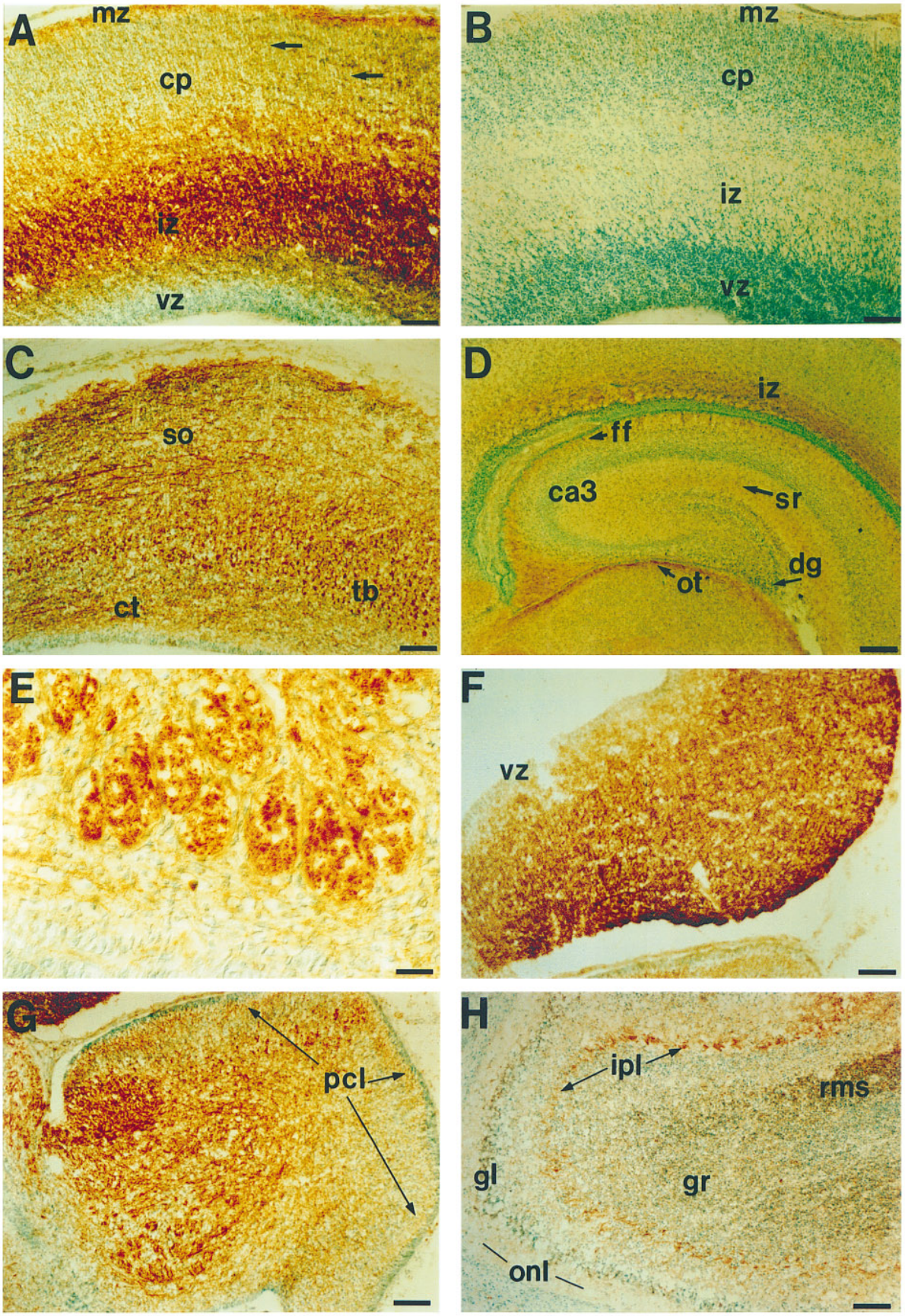
FIG. 3. Immunohistochemical localization of cerebroglycan in a Postnatal Day 0 rat head. A frozen section through a P0 rat head was immunostained for cerebroglycan and visualized with HRP and DAB and counterstained with methyl green. Immunoreactivity is seen in many regions of the CNS including the cortex (ctx), optic nerve and tract (o), anterior commissure (ac), fornix (f), cerebellum (cb), midbrain (mb), and inferior (ic) and superior (sc) colliculi. No immunoreactivity is seen outside of the nervous system. Bar, 1 mm.

than in most parts of the central nervous system (Altman and Bayer, 1984), cerebroglycan mRNA appears before E12 and disappears by E19 (Stipp *et al.*, 1994). Figure 5 shows the localization of cerebroglycan immunoreactivity in the E14 and E18 spinal cord. At E14 (Fig. 5A), intense staining is seen on the axons of spinal commissural neurons approaching and crossing the floorplate, on motor axons leaving the ventral cord in the ventral motor roots, and on axons within both the central and the peripheral roots of sensory neurons of the dorsal root ganglia, especially as they enter the spinal cord in the dorsal root entry zones. Immunoreactivity is not seen in the ventricular zone of the spinal cord nor in the notocord. Thus, cerebroglycan is expressed on every major axon tract in the E14 spinal cord. A similar pattern of staining, albeit less intense, was also observed in the E12 spinal cord.

In contrast, by E18, the pattern of cerebroglycan immunoreactivity has changed dramatically (Fig. 5B), and no longer includes the axon pathways of primary motor neurons which have largely reached their targets by this stage (Altman and Bayer, 1984). Instead, immunoreactivity is seen in the dorsal, lateral, and ventral funiculi. Immunoreactivity observed in the dorsal funiculus correlates well with the growth of suprasegmental ascending collaterals of spinal sensory neurons within the developing propriospinal tract (Mirnics and Koerber, 1995). Many of these ascending fibers originating in the caudalmost regions of the spinal cord do not reach their targets in the hindbrain until P0. Immunoreactivity seen in the lateral and ventral funiculi is most likely due to descending motor axons of the reticulospinal and rubrospinal tracts. Axons of each of these tracts have begun to penetrate the spinal cord by E16 and have reached mid- to lower thoracic levels by E18 (Lakke and Marani, 1991; Kudo *et al.*, 1993).

Cerebroglycan Is Polarized to Axons in the Hilar Region of the Hippocampal Dentate Gyrus

Although cerebroglycan immunoreactivity is especially intense in developing fiber tracts, it does not necessarily follow that cerebroglycan is expressed more highly on axons than on other parts of the neuron. Because the density of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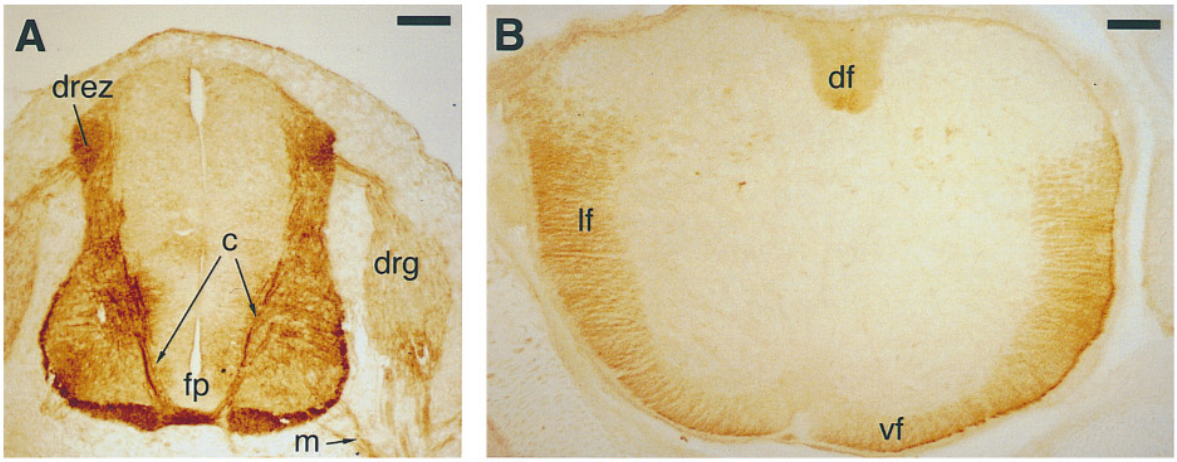

FIG. 5. Cerebroglycan expression in the developing rat spinal cord. Embryonic rats were immersion fixed in paraformaldehyde, cryoprotected in sucrose, sectioned in the coronal plane at 20 μm on a cryostat, and processed for cerebroglycan immunohistochemistry as described and photographed with brightfield optics. (A) At E14, cerebroglycan immunoreactivity is observed on all major axonal tracts present within the developing spinal cord, including spinal commissural axons (c), sensory axons of the dorsal root ganglia (drg)—which are especially prominent at the dorsal root entry zones (drez), and motor axons (m) exiting the cord within the ventral roots. (B) At E18, cerebroglycan immunoreactivity is greatly reduced throughout the spinal cord except in the dorsal (df), lateral (lf), and ventral (vf) funiculi where axons of the corticospinal tract are actively growing. Bar, 100 μm.

plasma membrane in axon bundles is much higher than in regions of neuronal cell bodies, immunostaining for proteins that are evenly distributed on neuronal plasma membranes can often appear much stronger on fiber tracts than around cell bodies (Morris and Grosveld, 1989). Levels of staining of dendritic fields should, in principle, be more comparable with that of axon bundles. However, because cerebroglycan is ubiquitously expressed in the developing nervous system, it is difficult to assess levels of dendritic staining, since it is difficult to find fields of dendrites that are not intermixed with potentially cerebroglycan-positive axons.

To circumvent these problems, we turned to the P21 hippocampus. This stage is long after the generation of most neurons and, accordingly, cerebroglycan expression in the brain as a whole is extremely low. In the hippocampus, however, production of new granule neurons continues in the dentate gyrus throughout life, albeit at a low level (Kaplan and Hinds, 1977; Bayer, 1980; Altman and Bayer, 1990a,b), so one might expect to see small numbers of hippocampal granule neurons expressing cerebroglycan even in adult animals. Conveniently, the axons and dendrites of dentate granule neurons extend from a compact layer of neuronal cell bodies and grow in opposite directions, with

FIG. 4. Immunohistochemical localization of cerebroglycan within the developing rat brain. Frozen sections of P0 rat brain were cut at 20 μm in the saggital plane except in D which is a coronal section. In each panel, rostral is to the left. All sections were counterstained with methyl green and photographed under brightfield illumination except E which was photographed using differential interference contrast microscopy. (A) The intermediate zone (iz) of the developing neocortex stains strongly for cerebroglycan as does the marginal zone (mz). Staining can also be seen on fibers extending through the cortical plate (cp). No staining is seen in the ventricular zone (vz). (B) A section through neocortex similar to A except that the primary antibody was omitted. (C) In the superior colliculus, cerebroglycan immunoreactivity is seen on optic axons within the stratum opticum (so), on tectobulbar (tb) axons seen in cross section in central tectal regions, and on corticotectal afferents (ct) in the deep tectal layers. (D) In the hippocampus, weak cerebroglycan immunoreactivity is seen in the mossy fiber layer in the hilar region of the dentate gyrus (dg), extending throughout the stratum radiatum (sr). Strong immunoreactivity is visible in the fimbria of the fornix (ff), as well as in the optic tract (ot) in the dorsal thalamus. (E) Large fascicles of axons within the posterior commissure stain strongly for cerebroglycan. (F) Within the caudal inferior colliculus, cerebroglycan immunoreactivity is widespread, absent only from the ventricular zone (vz). (G) In the cerebellum, strong immunoreactivity is seen throughout the deep cerebellar layers. Cerebroglycan is also seen in the Purkinje cell layer (pcl), where immunoreactivity is seen in dorsal, but not ventral regions. (H) In the olfactory bulb, the strongest cerebroglycan immunoreactivity is seen in the inner plexiform layer (ipl), presumably associated with mitral cell axons, as well as on presumptive granule neurons (gr) within the rostral migratory stream (rms). Weak immunoreactivity is also seen within the olfactory nerve (onl) and glomerular layers (gl). Bar in all panels, 100 μm except D (bar, 200 μm) and E (bar, 25 μm).

a single axon heading basally into the hilar region (to join other mossy fibers) and a single dendrite heading apically (Bayer, 1980; Gaarskjaer, 1986). Thus, at P21 it should be possible to individually examine cerebroglycan expression on well-separated axons, cell bodies, and dendrites of small numbers of granule neurons, without interfering immunostaining from any other cell type.

As shown in Fig. 6, this was indeed the case. In sections of the P21 hippocampus, small numbers of thin cerebroglycan-immunoreactive fibers were scattered throughout the hilar region; these fibers had the appearance of individual axons. In contrast, little if any cerebroglycan immunoreactivity was detected among the granule cell bodies nor among the layer containing their dendrites. These data demonstrate that, at least for some neurons, cerebroglycan is restricted to the axonal compartment and suggest that this polarized expression pattern may be a general feature of cerebroglycan expression.

Cerebroglycan Is Expressed on Neuronal Growth Cones

The strong localization of cerebroglycan to the axons of newly generated neurons raises the possibility that it could function on axonal growth cones. To address whether cerebroglycan is expressed on growth cones *in vitro* and *in vivo*, E18 retinal neurons were cultured on merosin-coated glass coverslips for 20 hr and immunostained for cerebroglycan. As can be seen in Fig. 7, strong cerebroglycan immunoreactivity was observed on growing neurites, including the growth cones. Heavy staining was seen along the leading edge of veil-rich regions (Fig. 7A), as well as along filopodia, where highly punctate staining (Figs. 7B and 7C), consistent with clustering of cerebroglycan in the membrane, was typically observed.

To determine if cerebroglycan is also a prominent component of growth cones *in vivo*, total proteoglycans were purified from freshly isolated growth cone particles and radioiodinated, and their core proteins were analyzed by SDS–PAGE following digestion with specific GAG lyases (Fig. 8A). Three major HSPGs were seen in these preparations, with apparent core protein molecular weights of approximately 135, 60, and 54 kDa, corresponding to syndecan 3, glypican, and cerebroglycan (formerly known as HSPGs M7, M12, and M13, respectively; Herndon and Lander, 1990). Identical results were obtained in five independent preparations. The identification of the 60- and 54-kDa species as glypican and cerebroglycan, respectively, was based on the ability of anti-glypican and anti-cerebroglycan antisera to specifically immunoprecipitate these molecules (Fig. 8B). The identification of HSPG M7 as syndecan 3 was made on the basis of an anti-syndecan 3 antiserum (generously provided by Dr. M. Bernfield) to specifically immunoprecipitate the 135-kDa species (not shown). Although the relative proportions of cerebroglycan, glypican, and syndecan-3 in growth cone particles are similar to those in whole-brain membranes (cf. Herndon and Lander, 1990), PGs as a whole

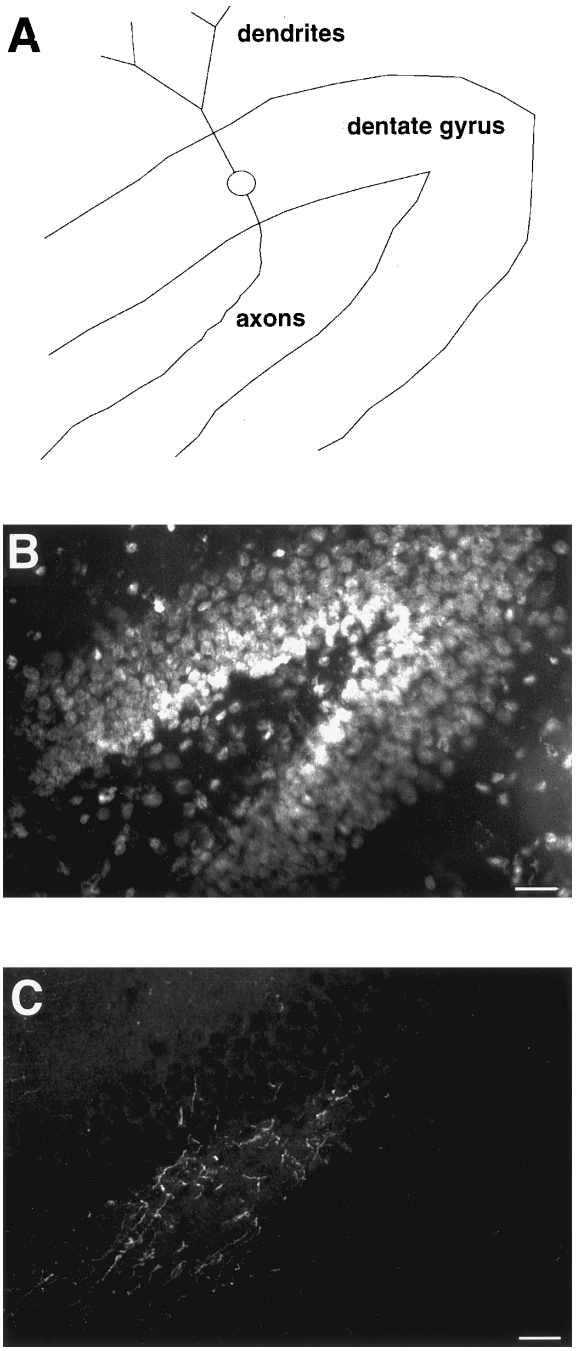

FIG. 6. Cerebroglycan expression in the hilar region of the Postnatal Day 21 rat hippocampus. Saggital sections of fresh-frozen P21 rat brain were cut at 12 μm on a cryostat, postfixed, and stained with the 521-2 antibody. Antibody binding was visualized using a Cy3-conjugated anti-rabbit second antibody, and the section was counterstained with bisbenzamide to allow visualization of nucleii. (A) Schematic of the cellular anatomy of the dentate gyrus. (B) Nuclei of granule neurons in the dentate gyrus labeled with bisbenzamide. (C) Cerebroglycan immunoreactivity demonstrated on mossy fibers by immunofluorescence. Bar, 40 μm.

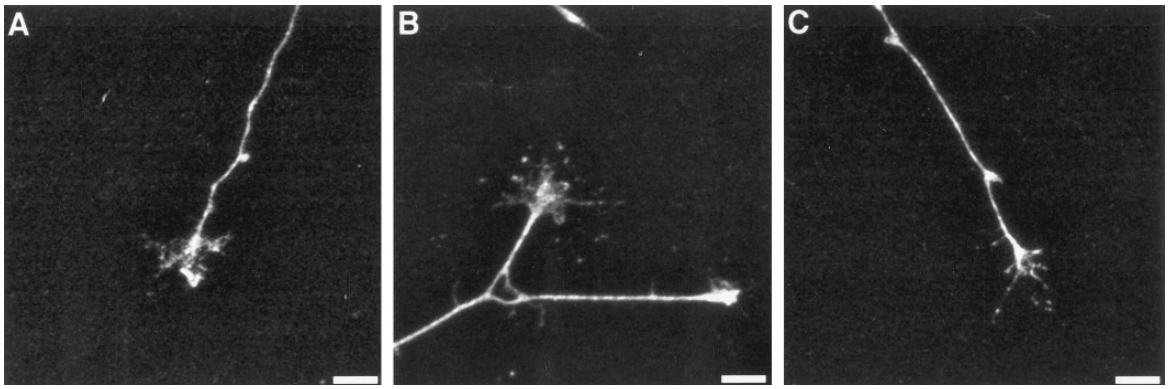

FIG. 7. Cerebroglycan expression on retinal growth cones *in vitro*. Retinal neurons from E18 rats were cultured overnight on merosin-coated glass coverslips. After fixation, they were immunostained for cerebroglycan, visualized using a Cy3-conjugated second antibody, and photographed at 63× under rhodamine fluorescence. Three separate examples of neurites bearing growth cones are shown in A, B, and C. The cell bodies from which these neurites extended were also immunoreactive (not shown). Bar, 15 μm.

were enriched in the growth cone fraction. Specifically, in growth cones of neonatal rat brain, PGs constituted between 1.21 and 1.69% of total protein (1.43 ± 0.24%, n = 3), whereas in whole-brain membranes they were approximately three times less abundant (0.45% of total protein, n = 2).

DISCUSSION

To investigate the roles of cell surface HSPGs in neural development, we generated specific antibodies against cerebroglycan, a GPI-anchored HSPG expressed specifically within the developing nervous system. The antibodies are directed against an epitope near the C-terminus of the mature polypeptide and recognize both the core protein and the intact HSPG (Figs. 1, 2, and 8). Immunostaining of the developing rat nervous system indicates that cerebroglycan is highly localized to developing axon tracts and growth cones. Furthermore, these data indicate that cerebroglycan is predominantly, if not exclusively, a product of neurons (rather than glial cells), a result that *in situ* hybridization data had suggested (Stipp *et al.*, 1994).

As long as they were examined at stages when axons are actively growing, all axon tracts appeared to be immunoreactive for cerebroglycan, suggesting that cerebroglycan may be a general marker expressed by immature neurons. In most cases, axon tracts became cerebroglycan negative within several days after the final mitosis of their neurons of origin (cf. Fig. 5), usually around the time that axons arrived at their targets. For instance, the axons of spinal sensory neurons, which reach their central targets between E16 and E18 (Mirnics and Koerber, 1995b), stained strongly for cerebroglycan at E14, but had largely lost cerebroglycan immunoreactivity by E18.

An apparent exception to the rule that cerebroglycan is only transiently expressed by growing axons is the lateral olfactory tract, which primarily consists of axons of the mitral cells of the olfactory bulb, but also contains fibers that originate in the raphe nuclei and locus coeruleus and innervate the olfactory bulb (Shipley and Ennis, 1996). Cerebroglycan immunoreactivity was detected in the lateral olfactory tract as late as P21 (unpublished observations), despite the fact that mitral cells are born between E14 and E16 (Bayer *et al.*, 1993) and raphe and locus coeruleus neurons are born by E16 and E14, respectively (Bayer *et al.*, 1993). Other than this, the only evidence of cerebroglycan expression at P21 was associated with regions of very late neurogenesis (dentate granule neurons of the hippocampus [Fig. 6]; granule neurons of the olfactory bulb [not shown]), or regions where small numbers of axons had not yet finished growing (e.g., the internal capsule, which contains some very late-developing axons of the corticospinal tract [not shown]).

In at least one cell type, the hippocampal granule neuron, expression of cerebroglycan is specifically polarized to the axonal compartment, as no dendritic or cell-body staining could be detected (Fig. 6). This could reflect either preferential sorting or preferential stabilization of the cerebroglycan molecule. In several types of cells, GPI–anchors have been shown to target proteins to specific cellular domains, including the axonal compartment of hippocampal pyramidal neurons *in vitro* (Dotti and Simons, 1990). Indeed axonin-1/TAG-1 is another example of a GPI-anchored glycoprotein that appears to be preferentially associated with axons (Dodd *et al.*, 1988). Nevertheless, it cannot be that GPI anchorage targets cerebroglycan or other proteins to axons, since the GPI-anchored glycoprotein Thy-1 is actually *excluded* from growing axons *in vivo* (Xue *et al.*, 1991). It will be interesting to determine whether particular structural features in the core protein and/or glycosaminoglycan chains of cerebroglycan play a direct role in controlling the unique localization of this proteoglycan.

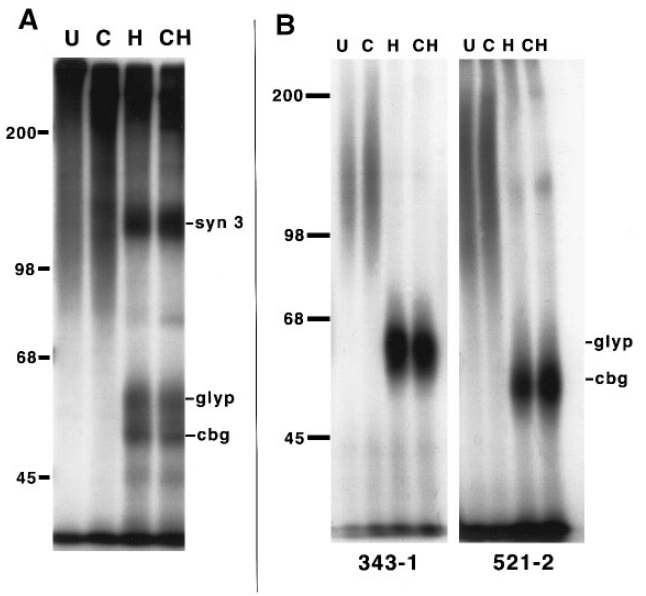

FIG. 8. Cerebroglycan expression in neonatal rat brain growth cone particles. (A) Total proteoglycans were isolated from P0 rat brain growth cone particles by ion-exchange chromatography, radioiodinated, digested with specific GAG lyases, and analyzed by SDS–PAGE on a 7.5% nonreducing gel. Lanes are untreated (U), chondroitinase ABC treated (C), heparitinase treated (H), and digested with both chondroitinase ABC and heparitinase (CH). Three distinct HSPGs are resolved following digestion with heparitinase, but not with chondroitinase. The core protein of syndecan 3 has an apparent MW of approximately 135 kDa, glypican has an apparent MW of 60 kDa, and cerebroglycan has an apparent MW of approximately 54 kDa. (B) Immunoprecipitation of glypican and cerebroglycan from radioiodinated growth cone proteoglycans. Immunoprecipitations were performed with anti-glypican (antibody 343-1) and anti-cerebroglycan (antibody 521-2) antisera as described under Methods, following digestion with GAG lyases as indicated. Immune complexes were eluted with hot SDS sample buffer and resolved by SDS–PAGE on a 7.5% nonreducing gel.

In a few cases, cerebroglycan staining in the P0 rat brain was not obviously associated with axon tracts: In the cerebellum, the strongest immunoreactivity was associated with regions where newly postmitotic neurons (deep cerebellar neurons, Purkinje cells) were completing cell migration (Fig. 4G). Similarly, in the P0 olfactory bulb, strong immunoreactivity was associated with the rostral migratory stream, the final stage of the migratory pathway for several types of olfactory interneurons (Fig. 4H). It is possible that the immunoreactivity seen in these locations represents staining of early axonal processes that are extended by cells as they migrate. More likely, in some neurons, cerebroglycan may initially be expressed diffusely, before becoming polarized to axons.

Potential Functions of Cerebroglycan

The data presented here indicate that, in most if not all of the central nervous system, cerebroglycan is expressed on axons from early stages of axon elongation to the time that synaptic contacts are initially made. This corresponds to the time when most of the navigational decisions of axons are being made. That cerebroglycan may play a role in these decisions is supported by the fact that this HSPG is highly localized to axons and, importantly, is expressed on growth cones, the organelles that are directly responsible for axonal responses to guidance cues. Interestingly, it appears that not just cerebroglycan, but all three of the major HSPGs of the developing brain—cerebroglycan, glypican, and syndecan-3—are prominent components of growth cones (cf. Fig. 8). In addition, immunoreactivity for both glypican (Karthikeyan *et al.*, 1994) and syndecan-3 (Gould *et al.*, 1995) has been seen on axon tracts. These observations suggest that cell surface HSPGs in general may play an important role in the responses of growth cones to their environment.

Currently, the most accepted model of how cell surface heparan sulfate proteoglycans function is that they act as coreceptors for a variety of secreted and cell-surface ligands. These include growth factors, such as the fibroblast growth factors, that require an interaction with heparin or heparan sulfate in order to bind to and initiate signaling from their receptors (Rapraeger *et al.*, 1991; Yayon *et al.*, 1991). They also include extracellular matrix proteins, such as fibronectins, laminins, and thrombospondins (Woods and Couchman, 1992). It is noteworthy that many of the molecules in these categories affect the behavior of growth cones and may serve as axon guidance cues *in vitro* and *in vivo* (Gomez and Letourneau, 1994; Kuhn *et al.*, 1995; McFarlane *et al.*, 1995). Whether cerebroglycan, or any other HSPG, is involved in growth cone responses to these molecules remains to be seen. However, it has been shown in some systems that removal of heparan sulfate (Wang and Denburg, 1992), or removal of GPI-anchored molecules (Chang *et al.*, 1992), can interfere with growth cone guidance *in vivo*.

To date, the most definitive data on the function of GPI-anchored HSPGs in the nervous system comes from the study of mutant alleles of the *Drosophila dally* (*division abnormally delayed*) gene, which encodes a proteoglycan of the glypican family that is approximately 45% similar to each of the known glypican family members, including cerebroglycan (Lander *et al.*, 1996; Nakato *et al.*, 1995). In flies homozygous for hypomorphic *dally* alleles, the lamina precursor cells, which give rise to synaptic target neurons for photoreceptor axons, show a delayed entry into mitosis in the first of two stereotypic cell divisions. The second cell division, which is triggered by an intercellular signal from the photoreceptor axons as they arrive in the target region, does not occur. Although the mechanism underlying this phenotype has yet to be elucidated, the results are consistent with a defect in a growth factor signaling pathway. It will be interesting to determine whether the dally protein acts as a coreceptor for any known *Drosophila* growth factors and, if so, whether vertebrate homologs of those growth factors also employ members of the glypican family as coreceptors.

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