

Neuronal Expression of Glypican, a Cell-Surface Glycosylphosphatidylinositol-anchored Heparan Sulfate Proteoglycan, in the Adult Rat Nervous System

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Cell-surface proteoglycans have been implicated in cell responses to growth factors, extracellular matrix, and cell adhesion molecules. M12, one of the most abundant membrane-associated proteoglycans in the adult rat brain, is a ~65 kDa glycosylphosphatidylinositol-linked protein that bears heparan sulfate chains (Herndon and Lander, 1990). To assess its identity, M12 was purified and internal peptide sequences obtained. Comparison of the results with protein sequence predicted by a cDNA cloned from PC12 cells indicated that M12 is rat glypican, a proteoglycan first cloned from human fibroblasts. In addition, antibodies raised against a rat glypican fusion protein specifically detected the 65 kDa brain proteoglycan core protein, both by immunoprecipitation and by Western blotting. Northern blot analysis using a rat glypican probe also detected glypican message in the adult, as well as the developing rat brain.

In situ hybridization with glypican RNA probes showed that glypican is expressed in a subset of structures in the adult rat nervous system. These include the hippocampus, dorsal thalamus, amygdala, cerebral cortex, piriform cortex, olfactory tubercle, several cranial nerve nuclei, the ventral horn of the spinal cord, and the dorsal root ganglia. Several other brain regions exhibited little or no hybridization over background. In most cases where glypican hybridization was observed, the signal could be localized specifically to the cell bodies of identifiable neurons, for example, spinal motoneurons, hippocampal pyramidal cells. In the cerebral cortex, glypican hybridization was found in layers 2/3, 5, and 6, but was missing from 1 and 4. The data suggest that glypican is expressed primarily by subpopulations of projection neurons in the adult rat nervous system.

[Key words: glypican, glycosylphosphatidylinositol, heparan sulfate proteoglycan, *in situ* hybridization, mRNA, neurons]

The interaction of cells with their environment is important for the growth and function of the nervous system. These interactions control processes such as cell adhesion, migration, prolif-

eration, axon growth and guidance, and responsiveness to growth factors. Proteoglycans (PGs), proteins that contain one or more covalently attached glycosaminoglycan (GAG) chains, are thought to be involved in many such interactions. PGs are found both in the extracellular matrix and on cell surfaces. Through their GAG chains PGs have the ability to bind a number of important nervous system proteins, including extracellular matrix proteins (such as laminin, fibronectin, thrombospondin, and agrin), cell-surface molecules (such as NCAM, myelin-associated glycoprotein, the amyloid- β -protein precursor), synaptic enzymes (AChE) and growth factors of the fibroblast growth factor (FGF) family (for reviews, see Jackson et al., 1991; Lander and Calof, 1993). In some cases it has been demonstrated that GAGs—in particular, GAGs of the heparan sulfate (HS) class—play an essential role in the functions of these proteins. For example, cell-surface HS is apparently required for basic FGF (bFGF) to bind to cells and exert its effects (Rapraeger et al., 1991; Yayon et al., 1991; Kan et al., 1993), as well as for NCAM-mediated cell adhesion (Cole et al., 1986; Reyes et al., 1990), and agrin-mediated clustering of muscle ACh receptors (Gordon et al., 1993).

Currently, it is not known which PGs in the nervous system mediate these and other biological activities, or where such PGs are expressed. Up to 25 PG core proteins have been reported in the developing and adult rat brain (Herndon and Lander, 1990), including molecules bearing both HS and chondroitin sulfate (CS) GAGs. Those PGs for which information exists on regional distribution in the nervous system are mainly CSPGs; these include the Cat-301 antigen (Hockfield et al., 1990), neurocan (Rauch et al., 1992), versican (Perides et al., 1992), and the T1 antigen (Iwata and Carlson, 1993)—all of which appear to be extracellular matrix-associated—and one cell surface CSPG, NG2 (Stallcup et al., 1983; Levine and Card, 1987; Nishiyama et al., 1991). In addition, immunological studies have identified distinct CS epitopes on subsets of CNS neurons (Fujita et al., 1989; Watanabe et al., 1989; Maeda et al., 1992).

In contrast, much less is known about the expression of PGs in the brain that bear HS chains. One, known as N-syndecan or syndecan-3 (for nomenclature, see Bernfield et al., 1992), is an integral membrane PG expressed mainly during perinatal development, and appears to be associated with many cell bodies (Carey et al., 1992). Other brain HSPGs have been characterized biochemically, but their distributions have not been elucidated (Margolis et al., 1975a,b; Klinger et al., 1985; Herndon and Lander, 1990; Nurcombe et al., 1993).

Two of the major HSPGs of the rat brain, known as M12 and M13 (Herndon and Lander, 1990), associate with isolated mem-

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branes and exhibit detergent-partitioning properties indicative of integral membrane proteins. These PGs lose their detergent-partitioning properties when treated with phosphoinositide-specific phospholipase C, indicating that they associate with membranes via a covalent glycosylphosphatidylinositol (GPI) lipid linkage (Herndon and Lander, 1990). Several other HSPGs have been reported to possess GPI anchors, including HSPGs isolated from rat ovarian granulosa cells (Yanagashita and McQuillan, 1989), mouse melanoma cells (Drake et al., 1992), rat Schwann cells (Carey and Stahl, 1990), and human lung fibroblasts (David et al., 1990). The latter molecule was cloned and given the name glypican (David et al., 1990).

In the present study we describe the purification and cDNA cloning of M12 from rat brain and show that it is the rat homolog of glypican. *In situ* hybridization studies are presented that indicate that M12/glypican is regionally expressed in the adult brain. Specifically, glypican is found to be expressed at high levels by restricted populations of projection neurons.

Some of these data have been presented previously in abstract form (Litwack and Lander, 1992).

Materials and Methods

Purification of HSPG M12. A membrane fraction was isolated from 46.4 gm wet weight of neonatal rat brains (roughly 165 animals), and a detergent extract of this fraction was prepared (Herndon and Lander, 1990). All further steps were done at 4°C except where noted. PGs were purified by loading the detergent extract onto a column (172 ml) of DEAE-Sephacel, washing sequentially in 50 mM Tris-HCl (pH 8.0 at 4°C), 0.1% Triton X-100, and protease inhibitors as in Herndon and Lander (1990) containing (1) 0.15 M NaCl (starting buffer), (2) 0.25 M NaCl, (3) 0.25 M NaCl, 6 M urea, and (4) 0.25 M NaCl, 6 M urea, with 50 mM formate (pH 3.5) replacing the Tris-HCl. The pH of the column was then restored to pH 8.0 with starting buffer, and the PGs were eluted with starting buffer containing 0.75 M NaCl. This PG-enriched fraction was concentrated and exchanged into starting buffer containing 0.15 M NaCl using a Centrprep-10 (Amicon), and was then digested with 0.09 U/ml chondroitinase ABC (Sigma) for 2 hr at 37°C. This material was loaded on a 0.2 ml DEAE-Sephacel column, washed with starting buffer (without protease inhibitors) and then 0.2 M NaCl, 6 M urea, 100 mM sodium acetate (pH 3.5), 0.1% Triton X-100. The column was washed with 25 mM ammonium acetate (pH 7.0), 0.1% Triton X-100 and eluted with a 20 ml 0.15 M–0.75 M NaCl gradient in this buffer. The fractions from 0.3 M to 0.75 M NaCl were pooled, and this PG-enriched fraction, now depleted of CSPGs, was concentrated and exchanged into 25 mM ammonium acetate (pH 7.0), 0.1% Triton X-100, 0.15 M NaCl in a Centricon-10. This sample was made 25 mM in Tris-HCl (pH 7.1 at 37°C) and digested with 9 µg/ml heparitinase (prepared as in Herndon and Lander, 1990) for 3 hr at 37°C. The sample was concentrated in a Centricon-10, subjected to electrophoresis in a 9% SDS-PAGE gel (Laemmli, 1970), and electroblotted to nitrocellulose (Schleicher and Schuell). HSPG core proteins were visualized by amido black staining (Schaffner and Weissman, 1973). M12 and M13 were excised, and were simultaneously eluted from the filters and digested with trypsin as in Tempst et al. (1990). The tryptic peptides were separated by reverse-phase HPLC (Tempst et al., 1990) and microsequenced by automated Edman degradation (Biopolymers Lab, MIT).

Cloning and sequencing of rat glypican. To obtain a human glypican probe, cDNA was synthesized from 0.5 µg of human foreskin fibroblast total RNA (purified as described by Chomczynski and Sacchi, 1987) using MMLV-reverse transcriptase (GIBCO–Bethesda Research Labs). This cDNA was then amplified by PCR using 120 pmol each of oligonucleotides GLA (5'-gggtccgaaagtgcctcagtc-3') and GLB (5'-gggtgtgctgctggtggccag-3'). These primers correspond respectively to bases 920–941 (in the sense orientation) and 1539–1518 (in the antisense orientation) of human glypican (David et al., 1990). PCR was performed for 30 cycles: 94°C for 30 sec, 55°C for 1 min, and 72°C for 1.5 min. The resulting product was digested with XhoI and KpnI (human glypican contains an XhoI site at base 690 and a KpnI site at base 1494), isolated by agarose gel electrophoresis, and cloned into pBluescript (Stratagene). The clone was verified by partial sequencing.

A Lambda-Gem 2 cDNA library (gift of Dr. John Wagner, Cornell University Medical School) constructed from A126 cells (a PC12 rat pheochromocytoma cell variant) was screened with the human glypican fragment labeled by the random primer method. Twelve positive clones were obtained from an initial screen of 400,000 plaques. The inserts from positive phage were subcloned into the EcoRI site of pBluescript and both strands of the largest, a 3 kb clone designated 41a, were sequenced by the dideoxynucleotide method using Sequenase (U.S. Biochemicals) or the fmol DNA Sequencing System (Promega). Sequencing primers were synthesized based on internal sequence (Biopolymers Lab, MIT), or restriction fragments were subcloned into pBluescript and sequenced using T3 and T7 primers. Clone 41a contains 3000 base pairs (bp) of the 3' end of rat glypican.

In order to obtain the 5' fragment of rat glypican, a primer-extended cDNA library was constructed from 5 µg of PC12 polyA⁺ RNA using the Time-Saver kit (Pharmacia). This polyA⁺ RNA was isolated over oligo-dT cellulose using the FastTrack kit (Invitrogen). The oligonucleotide RG6 (5'-ccctgcacaaaggatcgt-3', situated 213 bp downstream of the 5' end of clone 41a) was used to prime first-strand synthesis. The final cDNA product was ligated into the EcoRI site of pBluescript and transformed into XL1-Blue cells. A clone of about 950 bp, designated 7a3, was obtained by colony hybridization with probe 4EX (see below). Both strands of 7a3 were sequenced as above, and assembled with 41a based on their overlapping sequence. The assembled cDNA contains an open reading frame that predicts the glypican amino acid sequence.

In order to obtain glypican cDNA fragments directly from rat brain, PCR was performed as above from 0.5 µg of rat brain total RNA using the following sets of oligonucleotides: (1) RG15 (5'-tttggctcctccctcctcgt-3') and RG12 (5'-acgtcgtcctcaggctaaag-3'), (2) RG11 (5'-cactgccgtcactcgtg-3') and RG10 (5'-tgctcaaaaggctcctg-3'), (3) RG11 and RG4 (5'-ctcatcactgacaagtc-3'), and (4) RG5 (5'-aggatgccagtgtgacg-3') and RG7b (5'-tggccaagacagctctt-3'). The PCR products were purified on a 2% Nusieve (FMC) agarose gel, and sequenced with Taq polymerase using the fmol sequencing kit (Promega), and oligonucleotides end-labeled with ³²P-ATP.

Rat glypican probes. Probe 4EX is a 162 bp EcoRI-XhoI fragment from clone 41a inserted into pBluescript. Antisense RNA probes were made by digesting the plasmid with EcoRI and transcribing with T7 RNA polymerase (Stratagene) in the presence of ³⁵S-UTP; sense RNA probes were synthesized by digesting with XhoI and transcribing with T3 RNA polymerase (Stratagene). Probe 4P1 is a 567 bp PstI fragment subcloned from 41a into pBluescript. Antisense RNA probes were made by digesting with EcoRI and transcribing with T7 RNA polymerase. Probe 4P2 is a 332 bp PstI fragment subcloned from 41a into pBluescript. Antisense RNA probes were made by digesting with BamHI and transcribing with T7 RNA polymerase. A rat glyceraldehyde phosphate dehydrogenase DNA probe was a gift of Dr. Timothy Hayes (NIH). Probes for Northern blots were labeled with ³²P-dCTP using the Random Primer Labeling Kit (U.S. Biochemicals), and denatured at 100°C for 7 min before use.

Northern blotting. Total RNA, purified as described by Chomczynski and Sacchi (1987), was separated by electrophoresis in a 1.2% agarose/6.4% formaldehyde gel and transferred to nitrocellulose by capillary blotting. The filter was air dried for at least 1 hr, and then vacuum baked for 2 hr at 80°C. Prehybridization was carried out at 42°C overnight in 50% formamide, 5× saline-sodium phosphate-EDTA (SSPE), 5× Denhardt's, 0.1% SDS, 0.2 mg/ml yeast RNA, and 0.1 mg/ml salmon sperm DNA. Hybridization was carried out at 42°C for 2 d in 50% formamide, 5× SSPE, 1× Denhardt's, 0.1% SDS, and 0.1 mg/ml yeast RNA. Filters were washed once at 42°C in 50% formamide, 5× SSPE, 0.5% SDS for 30 min, twice at 42°C in 1× SSPE, 0.5% SDS for 15 min each, and twice at 65°C in 0.1× SSPE, 0.5% SDS for 30 min each. Filters were exposed to Kodak XAR-5 film at -80°C and/or imaged using a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

In situ hybridization. Brains from 6–7-week-old female Sprague-Dawley rats were dissected and quick frozen in isopentane chilled on dry ice. Cryostat sections (10 µm) were thawed onto gelatin-coated slides or Probe-On Plus slides (Fisher). Sections were stored at -80°C until use.

In situ hybridization was performed as described by Simmons et al. (1989). Briefly, sections were pretreated by fixation with 4% paraformaldehyde in PBS for 5 min, washed in 2× saline-sodium citrate (SSC), and treated with acetic anhydride to block positive charges. Sections were then washed again in 2× SSC, dehydrated stepwise in ascending alcohol concentrations, delipidated (in some instances) in chloroform for 10 min, washed again in 100% ethanol, and dried for at least 1 hr.

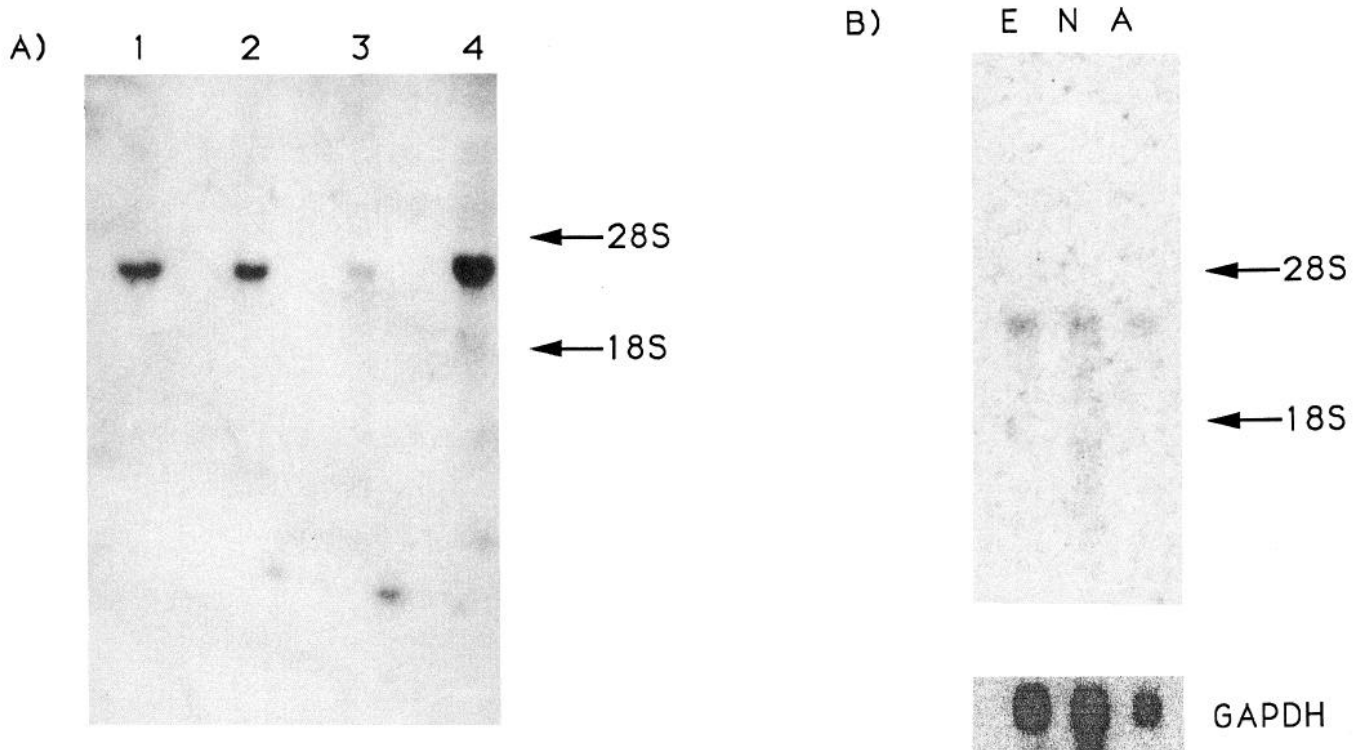


Figure 1. Detection of glypican message in rat brain, PC12 cells, and fibroblasts. *A*, Northern blot of 30 μ g of total RNA probed with a human glypican cDNA. *Lane 1*, Rat fibroblast cell line F2408 RNA; *lane 2*, PC12 cell RNA; *lane 3*, neonatal rat brain RNA; *lane 4*, human foreskin fibroblast RNA. *B*, Northern blot of 20 μ g of total RNA from rat brain probed with rat glypican cDNA probe 4X1. *Lane E*, embryonic day 18 rat brain RNA; *lane N*, neonatal rat brain RNA; *lane A*, adult rat brain RNA. The filter was stripped and reprobed for rat glyceraldehyde phosphate dehydrogenase (shown below), to demonstrate the efficiency of loading and transfer of RNA. Levels of glypican RNA relative to this control RNA are roughly equivalent at the three developmental stages. *Arrows in A and B* point to position of 18S and 28S ribosomal RNA bands.

Sections were incubated overnight at 55–60°C in 50% formamide, 10% dextran sulfate, 0.3 M NaCl, 1 \times Denhardt's solution, 1 mM EDTA, 10 mM Tris (pH 8.0), 0.1 mg/ml yeast tRNA, 200 mM DTT, 1% sarcosyl, and RNA probes at a concentration of 5×10^6 to 1×10^7 cpm/ml.

Sections were washed several times in 4 \times SSC, treated with RNase A for 30–60 min, washed in descending salt concentrations, and incubated in 0.1 \times SSC at 60°C for 30 min. Sections were then dehydrated in ethanol and dried. Sections were exposed to Hyperfilm- β Max (Amersham) or XAR-5 film (Kodak) for 10–14 d at -80°C . Sections were dipped in NTB-2 diluted 1:1 in water, stored at -80°C for 15–30 d, developed in D-19 (Kodak), counterstained with either cresyl violet or bisbenzimidazole (Hoechst 33258; Sigma), and studied with dark-field optics on a Zeiss Axiophot or WILD microscope. Hybridized sections were compared in some cases with nearby sections stained with cresyl violet, and with sections illustrated by Paxinos and Watson (1986) and Swanson (1992).

Production of glypican polyclonal antibodies. The EcoRI fragment of glypican clone 41a was cloned into the EcoRI site of pMalc2 (New England Biolabs), producing a construct that encoded a fusion protein of glypican with the bacterial maltose-binding protein. The construct was sequenced to verify that the junction was in-frame. The fusion protein was induced by IPTG, and isolated by affinity chromatography on an amylose column (New England Biolabs). The fusion protein was then dialyzed into 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 25 mM octyl- β -D-glucopyranoside. Antibodies were raised at Pine Acre Rabbitry and Farms (Norton, MA). Rabbits were injected intradermally with 0.5 mg of fusion protein in complete Freund's adjuvant and boosted subcutaneously with 0.5 mg of fusion protein in incomplete Freund's adjuvant. After two boosts, sera were collected and antibodies were purified over fusion protein coupled to Affi-Gel 10 (Bio-Rad).

Immunoprecipitation. Embryonic day 18 and adult rat brain membrane PGs were prepared and radioiodinated as described by Herndon and Lander (1990) and boiled in 0.1% SDS to disrupt aggregates of PGs that are present (cf. Herndon and Lander, 1990); Triton X-100 was then added to a final concentration of 1%. This material was incubated overnight at 4°C with 5 μ g of affinity-purified anti-glypican antibodies,

and then absorbed to protein A-Sepharose (Sigma) for 1 hr at 4°C. The protein A-Sepharose was then washed sequentially in 0.1 M Tris (pH 7.5), 2 mM EDTA, 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfate (CHAPS) containing (1) 150 mM NaCl, (2) 0.5 M NaCl, and (3) 150 mM NaCl; each wash was done at 4°C for 30 min. Antibody-antigen complexes were dissociated by boiling the Sepharose in 0.5% SDS and diluting into 1% Triton X-100, 50 mM Tris, 15 mM phosphoric acid (pH 7.3), and protease inhibitors. Aliquots were digested with heparitinase at a concentration of 2 μ g/ml at 43°C for 3 hr. Undigested and heparitinase digested samples were separated by electrophoresis on

Table 1. Sequence of tryptic peptides of purified M12 compared with human glypican

Peptide 15	AEALRPFPGDAPR
Human glypican	AEALRPFPGDAPR
Peptide 29	BXLPEVMGDLANQGXNPEVD
Human glypican	RYLPEVMGDLANQINNPEVE
Peptide 15a	MELETALHDSSR
Human glypican	AELETALRDSSR
Peptide 26	BALQATLATQLHGIDDFQ
Human glypican	RVLQAMLATQLRSFDDHFQ

Solid lines represent identities; dotted lines represent amino acids with similar properties. B refers to either K or R.

MELRARGWLLCAAALVACARGDPASKSRSCSEVRQIYGAKGFSLSVDPQAEISGEHLRI⁶¹ (rat)
 -----G----- (human)
 CPOGYTCCTSEMEENLANHSRMELETALHDSSRALOATLATOLHGIDDHFORLLNDSERTL¹²²
 -----R-HA-----R---V---M-----RSF-----H-----
 QDAFFGAFGDLYTONTRAFRDLYAELRLYRGANLHLEETLAEFWARLLERLFKQLHPQLL¹⁸³
 -AT-----E-----A-----S-----
 LPDDYLDCLGKQAEALRPFGDAPRELRLRATRAFVAARSFVQGLGVASDVVRKVAQVPLAP²⁴⁴
 -----E-----G-----
 ECSRAVMKLVYCAHCRGVPGARPCPDYCRNVLKGCLANQADLDAEWRNLLDSMVLITDKFW³⁰⁵
 -----L-----
 GPSGAENVIGSVHMWLAEAINALQDNKDTLTAKVIQCGNPKVNPBGSGPEEKRRRGKLAL³⁶⁶
 -T--V-S-----T-----R-----Q-P-----P
 QEKSSGTLEKLVSEAKAQLRDIQDYWISLPGTLCSEKMAMSPASDDRCWNGISKGRYLPE⁴²⁷
 R-RPPS-----V-----L-T-----MAR-----
VMGDGLANOINNPEVEVDITKPDMTIRQQIMQLKIMTNRLRGAYGGNDVDFQDASDDGSGS⁴⁸⁸
 -----S--N-----
 GSGGGCPDDACGRRVSKKSSSRTPLTHALPGLSEQEGQKTSAATRPEPHYFFLLFLFTLV⁵⁴⁹
 ---D--L--L---K--R-----SC-Q-PT-L-PL-LF-A
 LAAARPRWR⁵⁵⁸
 -TV-----

Figure 2. Comparison of rat and human glypican predicted protein sequences. The rat sequence is listed on *top*; the human, on the *bottom*. Amino acids in the human sequence identical to those in the rat sequence are indicated with a *dash*; differences are listed. Potential glycosaminoglycan attachment sites are indicated in *boldface*; potential *N*-linked glycosylation sites are indicated by *asterisks*; potential glypiation site is indicated by an *arrow*; putative *N*- and *C*-terminal transmembrane hydrophobic stretches (corresponding respectively to putative signal and glypiation sequences) are indicated in *italic*; regions corresponding to microsequenced peptides derived from purified M12 are *underlined*. The nucleotide sequence of the full-length cDNA extends about 70 bp past the 5' end and 200 bases beyond the 3' end of the cDNA reported by Karthikeyan et al. (1992). Sequence was assembled from clones 7a3 and 41a. The 3' end of clone 7a3 corresponds to base 883 and the 5' end of 41a corresponds to base 681 of the sequence in Karthikeyan et al. (1992).

a 10% SDS-PAGE gel. The gel was dried and exposed to Kodak XAR-5 film at -80°C .

Results

To determine the identities of two rat brain PGs that had previously been shown to contain GPI anchors, HSPGs M12 (core $M_r \sim 65$ kDa) and M13 ($M_r \sim 55$ kDa) (Herndon and Lander, 1990) were purified from neonatal rat brain, and peptides derived from the two core proteins were sequenced. Four M12-derived peptides were obtained. These possessed a total of about 80% identity to regions of the human glypican protein (Table 1), suggesting that M12 is rat glypican. M13 peptides were not highly similar to human glypican, and were used to isolate a cDNA clone encoding a novel PG core protein (Stipp et al., 1994).

To obtain a rat glypican cDNA clone with which to compare the M12 peptides, a human glypican probe was synthesized via PCR. This probe detected a single 3.7 kb message in neonatal rat brain total RNA (Fig. 1A). A message of the same size was detected in human foreskin fibroblast RNA (Fig. 1A), in agree-

ment with the previously reported human glypican message size from human lung fibroblasts (David et al., 1990). A single message of this size was also detected in PC12 cells, a rat pheochromocytoma cell line, and was more abundant in RNA from these cells than in RNA from neonatal rat brain (Fig. 1A). This finding is consistent with previous evidence that an M12-like proteoglycan is the major, if not only, heparan sulfate proteoglycan found in PC12 cells (Herndon et al., 1991).

A full-length rat glypican clone was obtained from PC12 cells as described in Materials and Methods. The cDNA sequence predicts a protein that is 89% identical to human glypican (the degree of identity increases to 91% if GPI-attachment sequences, which should not be present in the mature protein, are removed). The predicted protein sequence also contains sequences identical to those of the peptides derived from M12 (Fig. 2). Antibodies raised and purified against a fusion protein synthesized from part of this cDNA detected M12 in a Western blot of rat brain membranes (data not shown) and also immunoprecipitated M12 from crude mixtures of PGs obtained from both embryonic and adult rat brain (Fig. 3). Thus, we conclude that M12 is the rat form of glypican.

While this work was in progress, peptide sequences from a rat brain HSPG that were closely related to human glypican were reported (Karthikeyan et al., 1992). This report also contained a sequence for a rat glypican cDNA that contains four amino acid differences (due to single base differences) from the sequence presented here (Fig. 2): T instead of A at position 21, Y instead of N at position 312, A instead of G at position 362, and I instead of T at position 515. To address whether these differences represent sequence polymorphisms between rat glypican from PC12 cells and glypican from Sprague-Dawley rats, we used PCR to obtain, from Sprague-Dawley rat brain RNA, cDNA fragments spanning the regions of sequence discrepancy. Sequenceable PCR products were obtained that span three of the four regions (positions 312, 362, and 515), and direct sequencing of these fragments gave results that agreed in all cases with the sequence reported here for PC12 cells (data not shown). Consequently, the data do not support the existence of glypican sequence polymorphisms.

A Northern blot using a probe from the rat glypican cDNA confirmed that a single 3.7 kb glypican message is present in total RNA from adult rat brain, and also detected a similarly sized message in the embryonic (day 18) and neonatal rat brain (Fig. 1B). This is in agreement with the expression of M12 protein, which was detected at each of these stages (Herndon and Lander, 1990).

In situ hybridization experiments were undertaken to identify the cell types that express glypican in the adult rat nervous system. High levels of glypican expression were detected in several structures in the adult rat brain. In the hippocampus, glypican was expressed in high levels in the pyramidal cell layer (Fig. 4A). Furthermore, the pyramidal cell layer in regions CA3 and CA4 expressed glypican mRNA at substantially higher levels than in regions CA1 and CA2. Some hybridization was also seen to the dentate gyrus, although at lower levels than in regions CA3 and CA4. High levels of expression were also found throughout the dorsal thalamus (Fig. 4A-C), including the ventrolateral, ventrobasal, mediodorsal, lateral geniculate, and medial geniculate nuclei, but expression was not seen in the medial and lateral habenula nor in the ventral thalamus (e.g., the reticular nucleus and zona incerta).

Strong hybridization was also seen to certain nuclei of the amygdala (Fig. 4A,B), notably the pyramidal layer of the nucleus of the lateral olfactory tract and the basolateral nucleus. Weaker hybridization was seen to the lateral and basomedial nuclei, but no hybridization to the anterior, medial, central, and cortical

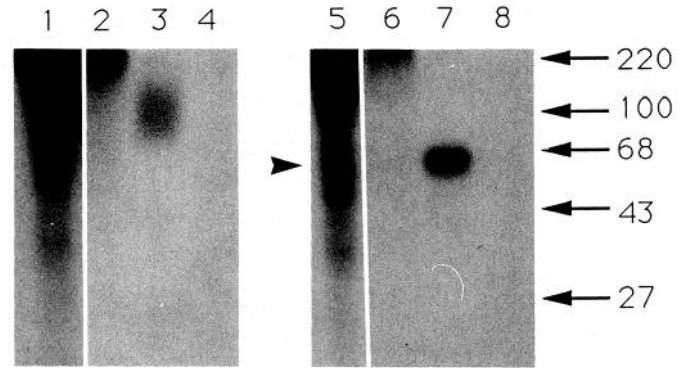


Figure 3. Antibodies raised against glypican fusion protein recognize adult rat brain HSPG M12. 125 I-labeled adult rat brain PGs (Herndon and Lander, 1990) were immunoprecipitated with 5 μ g of rabbit IgG (lanes 2 and 6), 5 μ g of affinity-purified anti-glypican serum (lanes 3 and 7), or 5 μ g of anti-glypican serum containing 20 μ g of glypican fusion protein. Lanes 1 and 5 show the starting material. Samples were either subjected to SDS-PAGE without heparitinase treatment (lanes 1-4) or treated with heparitinase before electrophoresis (lanes 5-8). Arrowhead indicates the position of the M12 core protein. *M*, markers (in kDa) are shown at right.

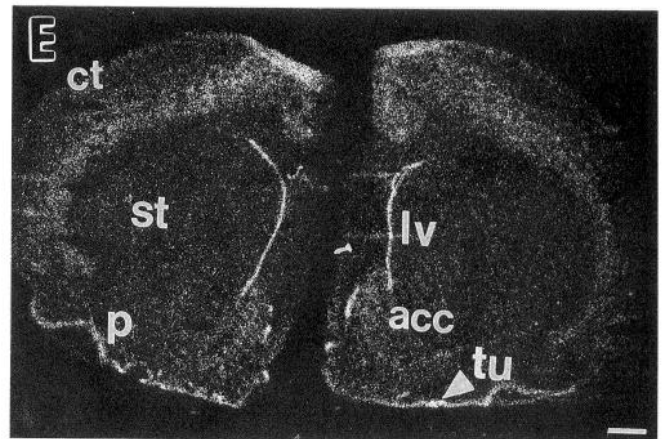
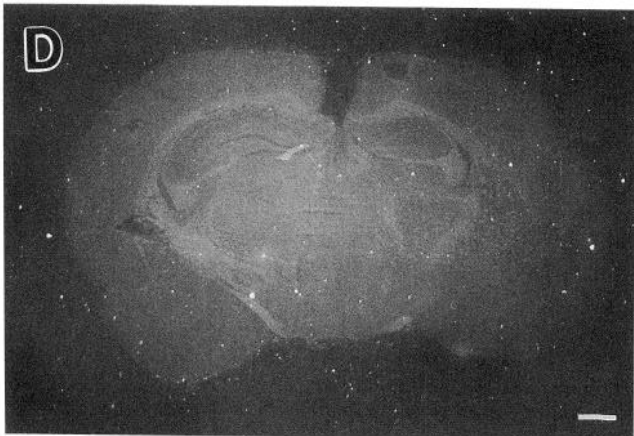
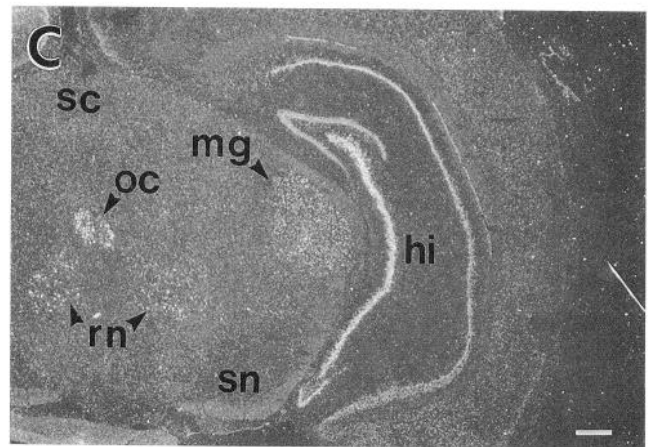
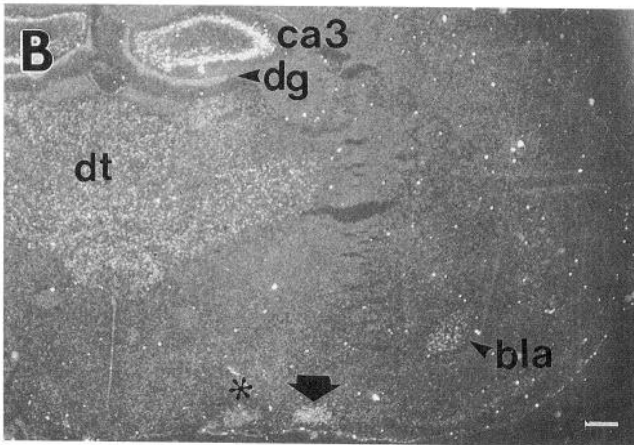
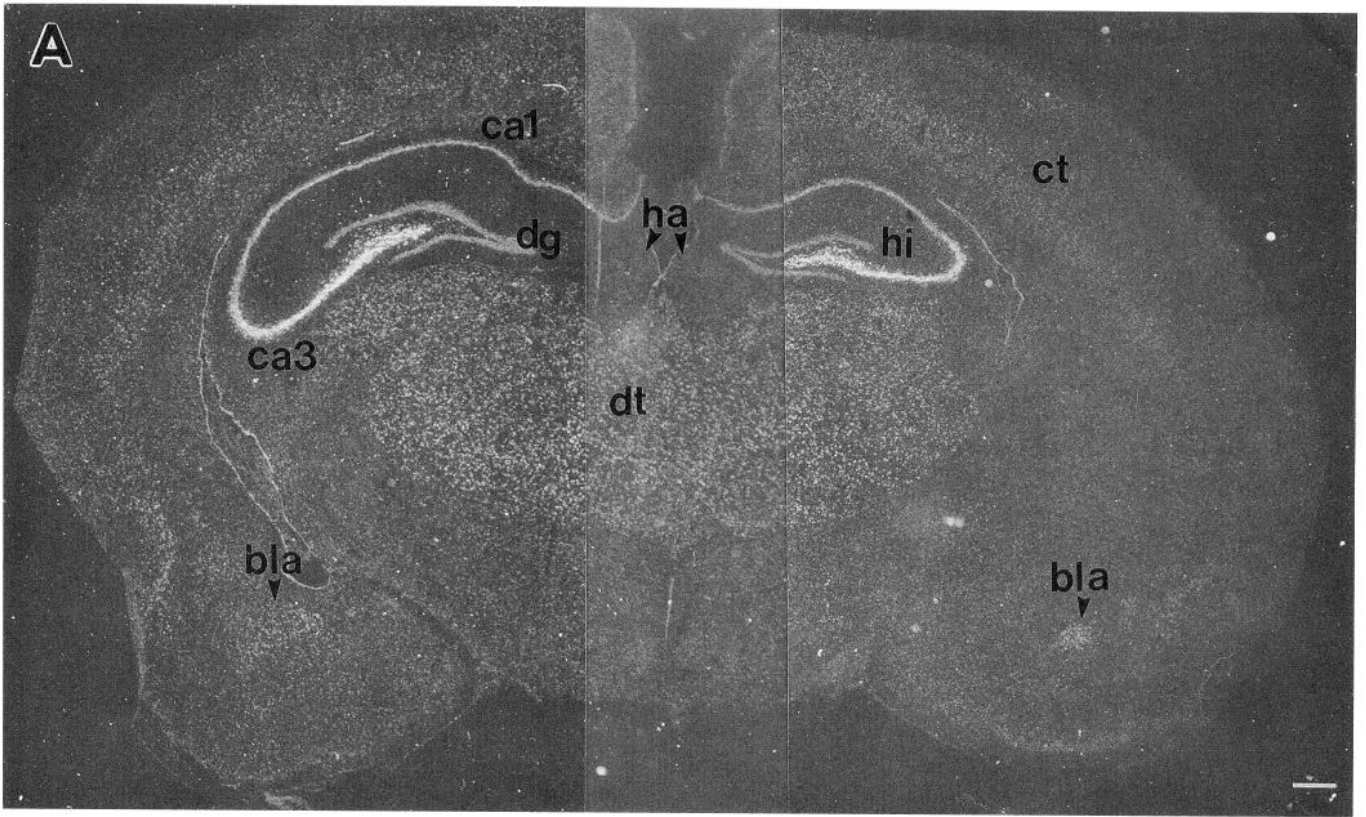
nuclei was detected. The sense strand of glypican did not hybridize to any of these or other structures (Fig. 4D). In addition, similar results were obtained using several antisense probes synthesized from different regions of glypican (see Materials and Methods) (data not shown).

Other forebrain structures tended to show somewhat less hybridization than that observed in the hippocampus, thalamus, and amygdala. These included the piriform cortex (Fig. 4E), olfactory tubercle (Fig. 4E), septal nuclei (data not shown), and the supraoptic (Fig. 4B) and paraventricular nuclei (data not shown) of the hypothalamus. Still weaker, but detectable hybridization was seen to the cerebral neocortex (Fig. 4E; see also below) and nucleus accumbens (Fig. 4E).

In the brainstem, relatively strong glypican hybridization was seen to the red nucleus (Fig. 4C), locus coeruleus (Fig. 4F,G), and several cranial nerve nuclei, including the motor nuclei of the 5th (4F,G), 7th (4H), and 12th (4I) nerves. Hybridization was also seen to the oculomotor complex (Fig. 4C). In the spinal cord, relatively strong hybridization was seen to the ventral horns (not shown), and to the dorsal root ganglia (Fig. 4J,K).

Many of the brain regions where glypican hybridization was seen contain principal neurons with large, easily recognizable

Figure 4. *In situ* hybridization of adult rat brain with rat glypican. *A*, Coronal forebrain section probed with 4X1, viewed in dark field. *B*, Coronal forebrain section at the level of the anterior thalamus probed simultaneously with 4X1 and 4P2, viewed in dark field. *C*, Coronal section through the posterior thalamus and anterior midbrain, probed with 4X1, viewed in dark field. *D*, Coronal section at the level of that in *A* probed with the sense (control) strand of 4X1, viewed in dark field. *E*, Coronal section through the anterior forebrain probed simultaneously with 4X1 and 4P2; hyperfilm image. *F*, Coronal section through the brainstem, probed with 4X1, viewed in dark field. *G*, A section approximately 30 μ m from that in *F* stained with cresyl violet. *H*, Coronal section through the brainstem, probed with 4X1, viewed in dark field. *I*, Coronal section through the brainstem, probed with 4X1, viewed in dark field. *J*, Section through a dorsal root ganglion, probed with 4P1, viewed in dark field. Arrows point to silver grains clustered over sensory neuron cell bodies. *K*, Same section as in *J* stained with cresyl violet. *L*, High-magnification view of the septum and lateral ventricle from a coronal section through the forebrain. *M*, Same section as in *L* stained with cresyl violet. Asterisk in *B* indicates supraoptic nucleus of the hypothalamus; arrow in *B* indicates the pyramidal layer of the nucleus of the lateral olfactory tract. *acc*, nucleus accumbens; *bla*, basolateral nucleus of the amygdala; *cb*, cerebellum; *ct*, cerebral cortex; *dg*, dentate gyrus; *dt*, dorsal thalamus; *e*, ependyma; *ha*, habenula; *hi*, hippocampus; *lc*, locus coeruleus; *lv*, lateral ventricle; *me5*, mesencephalic nucleus of the trigeminal (5th) nerve; *mg*, medial geniculate nucleus of the thalamus; *mo5*, motor nucleus of the trigeminal (5th) nerve; *oc*, Edinger-Westphal nucleus of the oculomotor complex; *p*, piriform cortex; *rn*, red nucleus; *s*, septal area; *sc*, superior colliculus; *sn*, substantia nigra; *st*, striatum; *s5*, sensory nucleus of the trigeminal (5th) nerve; *tu*, olfactory tubercle; *7*, nucleus of the 7th cranial nerve; *12*, nucleus of the 12th cranial nerve. In the sequence reported by Karthikeyan et al. (1992), 4X1 corresponds to bases 681-843, 4P1 corresponds to bases 1873-2440, and 4P2 corresponds to bases 2435-2767. Scale bars: *A-I*, 500 μ m; *J* and *K*, 200 μ m; *L* and *M*, 20 μ m.



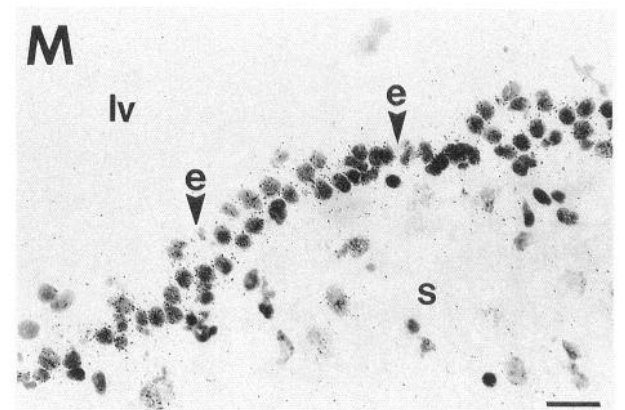
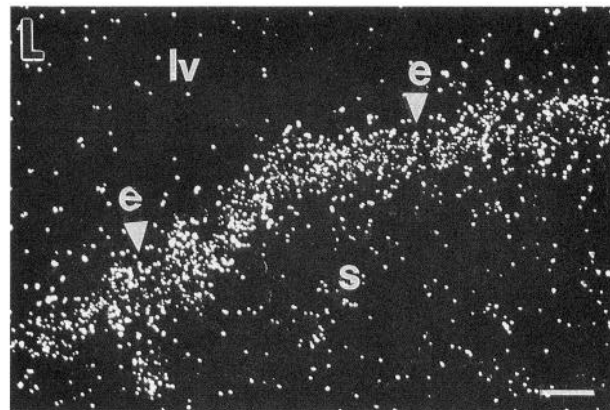
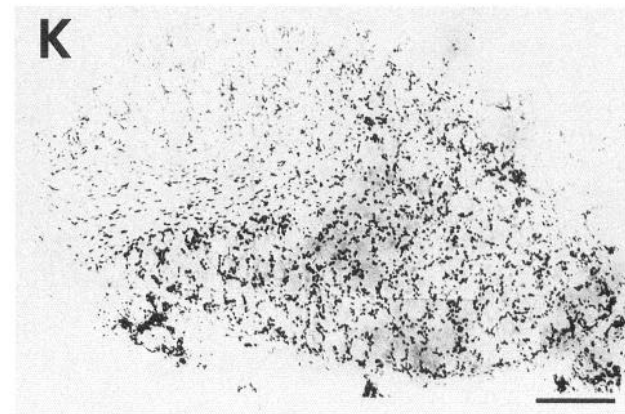
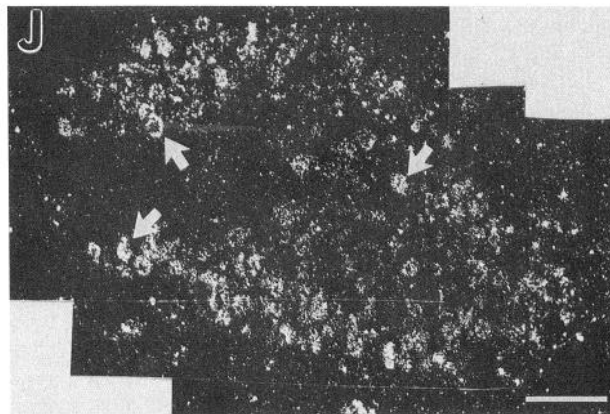
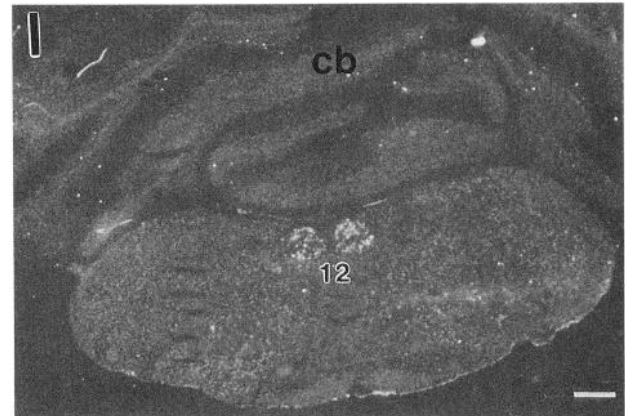
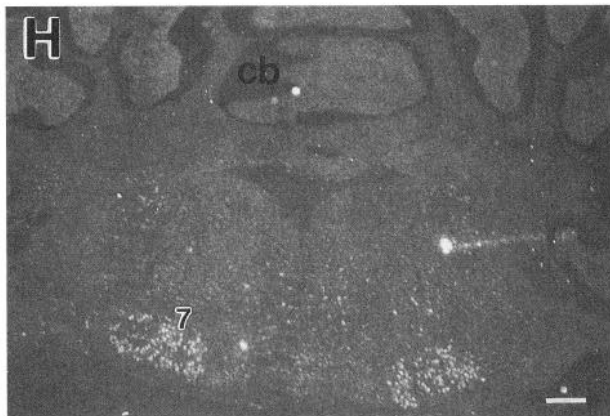
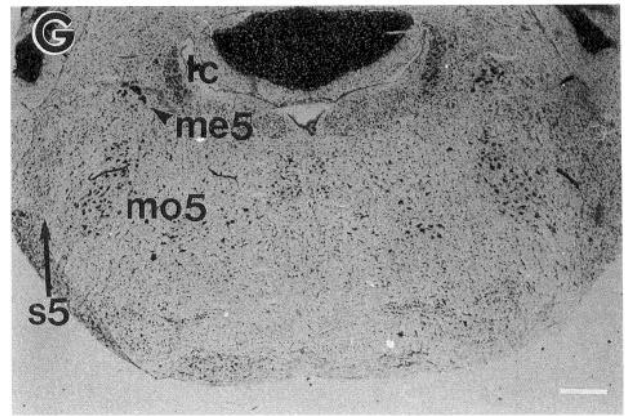
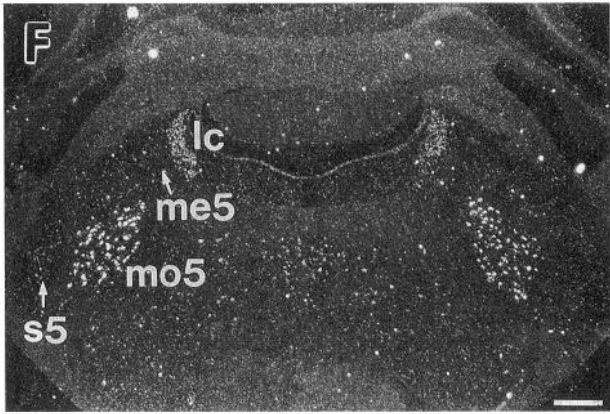


Table 2. Relative levels of glypican hybridization in the adult rat nervous system

Forebrain		
Cerebral neocortex		
Layers 2/3, 5, 6	+	
Layers 1, 4	-	
Hippocampus		
CA1, CA2	++	
CA3, CA4	+++	
Dentate gyrus	+	
Caudoputamen	-	
Dorsal thalamus		
Ventral anterior-lateral nucleus	++	
Ventral posterolateral nucleus	++	
Mediodorsal nucleus	++	
Nucleus reuniens	++	
Lateral geniculate nucleus	++	
Medial geniculate nucleus	++	
Anterodorsal	++	
Anteromedial	++	
Lateral dorsal	++	
Anteroventral nucleus	+	
Ventral thalamus		
Reticular nucleus	-	
Zona incerta	-	
Epithalamus		
Medial habenula	-	
Lateral habenula	-	
Amygdaloid nuclei		
Basolateral	++	
Nucleus of the lat. olfactory tract	++	
Lateral and basomedial	+	
Anterior, medial, central, cortical	-	
Hypothalamus		
Supraoptic nucleus	+	
Paraventricular nucleus	+	
Arcuate nucleus	-	
Piriform cortex	+	
Olfactory tubercle	+	
Septum	+	
Nucleus accumbens	+	
Endopiriform nucleus	+/-	
Ependyma	++	
Large meningeal blood vessels	+	
Brainstem		
Superior colliculus	-	
Inferior colliculus	-	
Red nucleus	++	
Substantia nigra	-	
Locus coeruleus	++	
Cerebellum		
Granule cell layer	-	
Purkinje cell layer	-	
Cranial nerve nuclei		
Edinger-Westphal	++	
Motor V	+++	
Motor VII	+++	
Motor XII	+++	
Mesencephalic V	+/-	
Sensory V	+/-	

Table 2. Continued

Spinal cord		
Ventral horn motoneurons		++
Dorsal horn neurons		+/-
Dorsal root ganglion neurons		++

Intensity of hybridization is graded as +++ (very strong), ++ (strong), + (moderate), +/- (weak), or - (absent), based in part on autoradiographic exposure times. For example, structures such as fields CA3 and CA4 of the hippocampus were darkly exposed in <15 d, while structures such as the cerebral cortex were usually only detected in longer exposures (≥ 25 d), or when two probes were used simultaneously during hybridization. Structures graded as - sometimes exhibited small clusters of silver grains over rare large cell bodies. Unlisted structures were not determined.

cell bodies. When sections containing these regions were examined at higher magnification, it could frequently be established that hybridization was localized to such neurons. For example, Figure 5*A* shows glypican hybridization to cells in the nucleus of the 5th cranial nerve. Figure 5*B* shows that clusters of silver grains are present over large, lightly stained motoneuron cell bodies, but not over smaller, more darkly stained cell bodies (i.e., glia and small neurons). Similar results, that is, hybridization to large, lightly stained cells, were obtained in other regions of the brain, such as the thalamus and hippocampus (data not shown).

A characteristic of most of the neurons in regions that were found to express glypican mRNA is that they are projection neurons. In the cerebral neocortex, however, large numbers of local circuit neurons are also present, and in layer 4 account for a large majority of the neurons (Zilles, 1990). Interestingly, glypican hybridization was not detected in layer 4 of the cortex, but was readily detected in other cell body layers, that is, 2/3, 5, and 6 (Fig. 6*A,B*). In layer 2/3, hybridization was strongest in the superficial part (Fig. 6*B*), and could be localized to a subpopulation of large cell bodies (Fig. 6*E,F*). In layers 5 and 6, hybridization was present at all levels, and was associated with most, but not all, large cell bodies (Fig. 6*C,D*). Although cell body size and location alone are not sufficient to establish the projection patterns of cortical neurons, the data are at least consistent with the view that glypican is primarily expressed by projection neurons.

Many types of projection neurons throughout the brain expressed little or no glypican mRNA, however. These included most neurons of the caudoputamen (Fig. 4*E*), the medial and lateral habenula (Fig. 4*A*), the superior and inferior colliculi (Fig. 4*C* and data not shown), the substantia nigra (Fig. 4*C*), and the cerebellum (Fig. 4*H,I*). In the latter structure, individual Purkinje cells are readily visualized, and could be clearly shown to exhibit no glypican hybridization over background (Fig. 5*C,D*). Although the possibility remains that levels of glypican mRNA too low to detect are present in these cells, the results emphasize the fact that levels of glypican mRNA expression throughout the brain do not merely correlate with cell body size.

In addition to being present in subsets of neurons, glypican mRNA was also found in some supporting cell (stromal) elements of the brain. For example, glypican hybridization could be seen associated with some large blood vessels, although at weaker levels than in many of the glypican-expressing neurons (data not shown). Higher but variable levels of hybridization could also be seen associated with ependymal zones (e.g., of the lateral ventricles) (Fig. 4*E,L,M*).

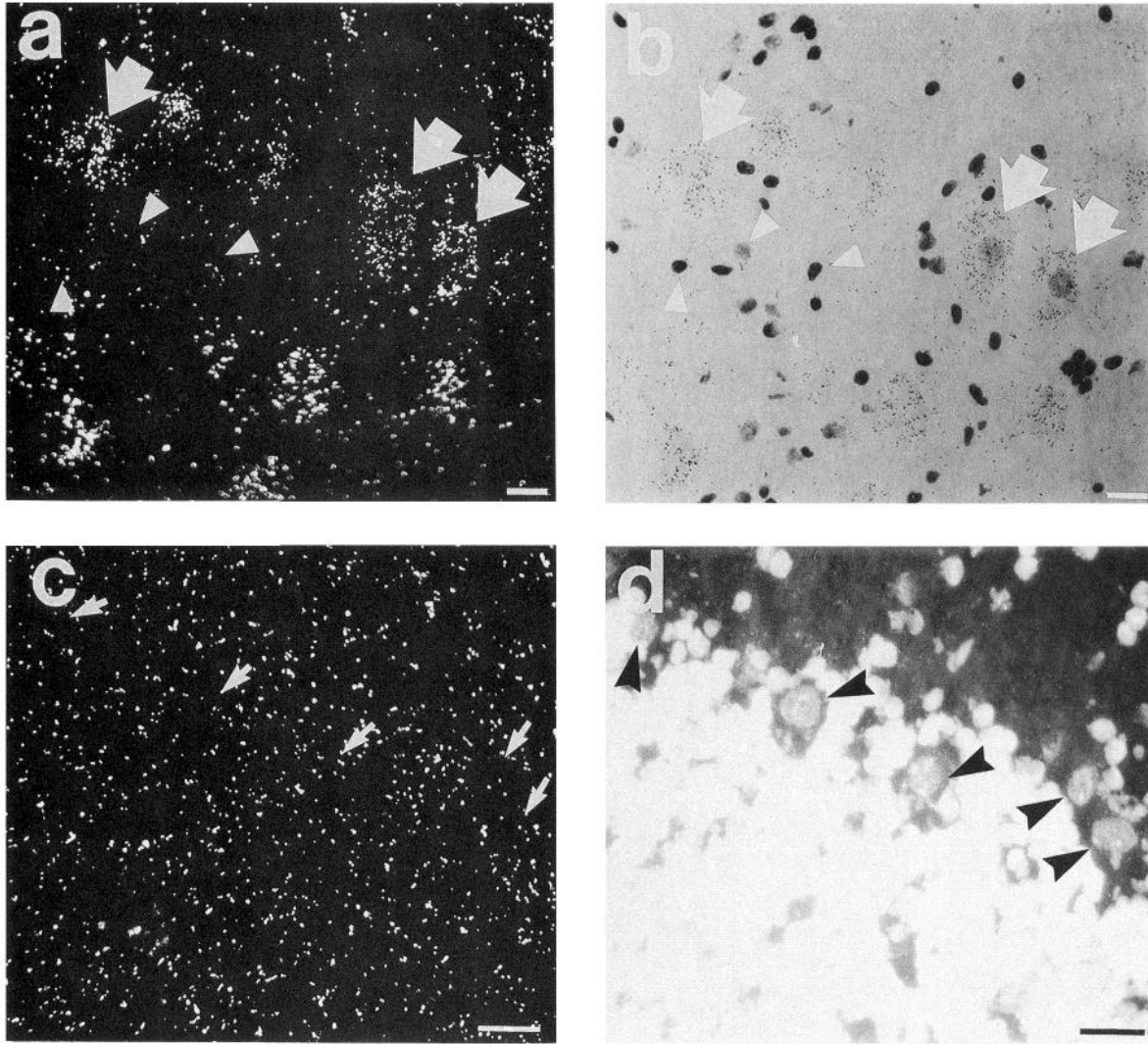


Figure 5. Neuronal expression of glypican. *A*, Motor nucleus of the trigeminal nerve. *Arrows* point to clusters of silver grains. *Arrowheads* point to positions of small nuclei. Dark-field view. *B*, Same field as in *A*. *Arrows* point to large, lightly stained nuclei under the clusters of silver grains indicated in *A*. *Arrowheads* point to small nuclei at positions indicated in *A*. *C*, Cerebellar cortex. *Arrows* point to positions of Purkinje cells. Dark-field view. *D*, Same field as in *C*. *Arrowheads* indicate the Purkinje cells marked in *C*. Scale bars, 20 μm .

Discussion

A major PG of the adult rat brain, HSPG M12, has been identified as glypican based on the following observations: (1) M12 is a GPI-anchored PG with a core protein size (~ 65 kDa) similar to that observed for human fibroblast glypican (David et al., 1990; Herndon and Lander, 1990), (2) peptides derived from M12 have amino acid sequences identical to those predicted from a rat glypican cDNA (this is in agreement with Karthikeyan et al., 1992, who also identified peptide sequences in a rat brain HSPG core protein that corresponded to sequences in a rat glypican cDNA), and (3) antibodies raised against a rat glypican fusion protein specifically immunoprecipitate M12 from a crude mixture of brain PGs and recognize a PG core protein that comigrates with M12 on a Western blot of heparitinase-digested brain membranes (data not shown). In addition, the expression of glypican message parallels the appearance of M12 core protein: we have found that glypican message is expressed in the adult and developing rat brain, as is M12, and that glypican mRNA can be detected by *in situ* hybridization in many brain regions.

Neuronal expression of glypican

The regional distribution of glypican mRNA in the adult brain, as revealed by *in situ* hybridization, suggests the following general features of glypican expression in the CNS.

Glypican is primarily a product of neurons. In many of the regions where glypican mRNA is expressed, neuronal cell bodies can be recognized by morphological criteria, for example, location, nuclear size, and intensity of cresyl violet staining. In such regions (e.g., hippocampus, motor nuclei, dorsal root ganglia), glypican hybridization is found directly over such cell bodies. This suggests that glial cells, which outnumber neurons in the brain, do not express glypican, although low level expression, or expression by small numbers of glia cannot be ruled out. Supporting and connective tissue elements of the brain (e.g., linings of large blood vessels, meninges, ependyma) also exhibit some glypican hybridization, although at relatively low levels compared with neuronal expression.

Glypican-expressing neurons are predominantly projection neurons. Each of the neuronal populations that expresses glypican appears to consist mainly of cells with axons that project

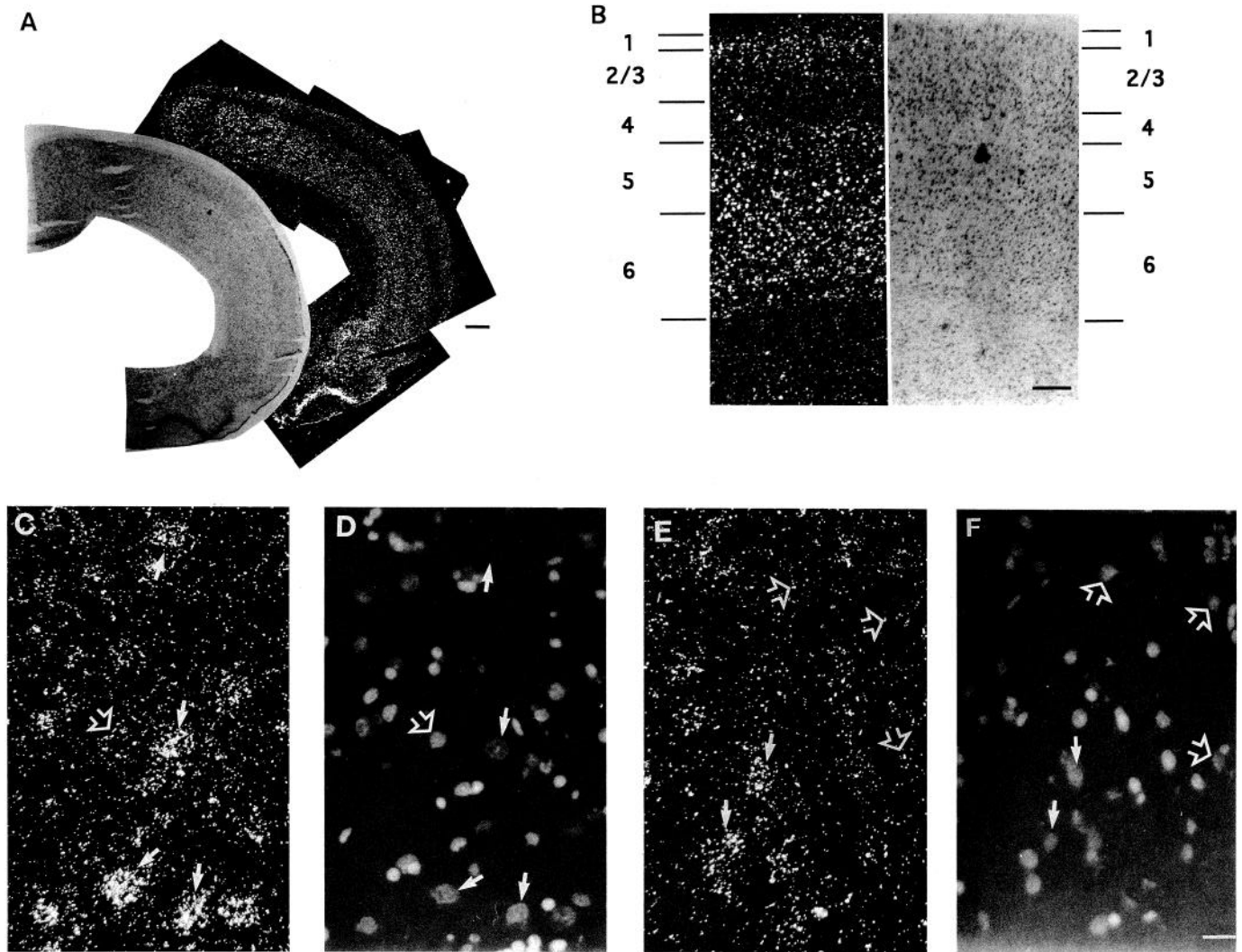


Figure 6. Glypican expression in the cerebral cortex. *A*: *Right*, Coronal section at the level of the basal ganglia stained with cresyl violet. *Left*, Dark-field view of coronal section 30 μm from that on left hybridized simultaneously with 4X1 and 4P1. *B*, Magnified views of corresponding regions from sections in *A*. *Lines* indicate approximate divisions between cortical layers. *C*, High-magnification view of layer 5, dark field. *D*, Same field as in *C*, stained with Hoechst 33258. *E*, High-magnification view of layer 2, dark field. *F*, Same field as in *E*, stained with Hoechst 33258. *Solid arrows* in *C–F* indicate clusters of silver grains, and the cell nuclei with which they are associated; *open arrows* indicate large cell bodies lacking clusters of silver grains. Scale bars: *A*, 500 μm ; *B*, 200 μm ; *F*, 20 μm .

to other brain regions, or, in the case of motoneurons, to the periphery. Even in the cerebral cortex, glypican expression is present in laminae with corticocortical or corticofugal connections (e.g., layers 2/3, 5, and 6), but absent from layer 4, which consists primarily of local circuit neurons. Since projection neurons typically have long axons, relatively high levels of glypican mRNA expression might be required in some projection neurons, if it turns out that the physiological processes that involve glypican require the molecule to be present at the axonal surface. Although it remains to be determined whether glypican is, in fact, expressed on the surfaces of axons, it is known that other GPI-anchored proteins are [e.g., F3/F11 (Faivre-Sarraillh, 1992), Tag-1 (Dodd et al., 1988; Furley et al., 1990), Thy-1 (Xue et al., 1990)].

Levels of glypican mRNA expression vary substantially among different populations of neurons. Based on the autoradiographic exposure times required to detect glypican expression in different brain regions, we can estimate relative levels of glypican

expression. High-level expression does not bear a simple relationship to neuronal cell body size or axon length. Indeed, some large and medium-sized projection neurons do not express detectable glypican (e.g., cerebellar Purkinje cells).

Although others have suggested that some PGs are localized to neurons that form functional or anatomical systems (Hockfield et al., 1990; Maeda et al., 1992), the types of neurons that express glypican mRNA do not correspond to any single functional or transmitter-specific subdivision of the brain. Nevertheless, limbic structures—hippocampus, amygdala, thalamus, cingulate and piriform cortex, nucleus accumbens, and septal nuclei—are relatively prominent among glypican-expressing structures. At least one other GPI-anchored protein is known to be associated with limbic system structures [LAMP (Levitt, 1984; Zacco et al., 1990; Zhukareva and Levitt, 1993)]. Also prominent among glypican-expressing neurons are neurons whose axons contribute to peripheral nerves (spinal and cranial motoneurons; dorsal root ganglion neurons).

Functional roles of glypican in the adult rat nervous system

Although glypican is only one of many PGs in the mammalian brain, there are reasons to believe that whatever roles it plays there may be unique. In adult brain, glypican is a major HSPG, and it is the only HSPG that exhibits detergent partitioning properties indicative of an integral membrane protein (Herndon and Lander, 1990). This suggests that the bulk of the HS borne by integral membrane proteins in the adult brain may be found on glypican. Since glypican is only expressed by certain populations of neurons, this suggestion raises the interesting possibility that CNS glia and some neurons may be relatively or entirely deficient in cell-surface HS. If so, expression patterns of glypican might play an important role in restricting the sites of action of molecules that normally interact with cell-surface HS.

Among such molecules that are found in the adult brain, members of the FGF family of polypeptide growth factors are present in many locations where glypican-expressing neurons are also found. Acidic FGF, for instance, is highly expressed in spinal and cranial motoneurons, as well as neurons of the dorsal root ganglia, thalamus, septum, and red nucleus, but differs from glypican in being largely absent from the cerebral cortex and hippocampus (Elde et al., 1991; Stock et al., 1992). However, hippocampal pyramidal neurons do express basic FGF [primarily in subfield CA2 (Gómez-Pinilla et al., 1992; Woodward et al., 1992)], and FGF-5 [in subfields CA2 and CA3 (Gómez-Pinilla and Cotman, 1992)]. Basic FGF is also found in some cranial motor nuclei (Gómez-Pinilla et al., 1992), and FGF-5 is present in skeletal muscle (Hughes et al., 1993). Although the functions of these growth factors in the adult nervous system are not known, evidence suggests that they may be involved in facilitating neuronal repair and/or neuronal survival (Gómez-Pinilla et al., 1992; Hughes et al., 1993). Since the actions of growth factors of the FGF family apparently require the participation of the HS chains of HSPGs (Rapraeger et al., 1991; Yayon et al., 1991), glypican may play an important role in the trophic and injury responses of neurons.

Glypican could also play a role in neuronal injury responses by virtue of interactions with HS-binding extracellular matrix molecules. For example, laminin, a strongly neurite outgrowth-promoting molecule, becomes exposed in the endoneurial sheaths of peripheral nerves during nerve regeneration (Bignami et al., 1984; Longo et al., 1984); both motoneurons and primary sensory neurons express glypican, which could then be involved in the recognition of and response to endoneurial laminin. Alternatively, it is known that at least one HSPG that is released by cells strongly inhibits the neurite outgrowth effects of laminin (Muir et al., 1989); neuronal glypican might be released under some circumstances and thereby interfere with regeneration. Significantly, GPI-anchored molecules, including some GPI-anchored PGs, are known to be shed by cells, presumably through the action of endogenous surface phospholipases (Ishihara et al., 1987; Carey and Evans, 1989; Low, 1989). This could account for the existence of an HSPG with biochemical properties very similar to those of glypican in the adult rat brain soluble (non-membrane-associated) fraction ("S9"; Herndon and Lander, 1990).

It is possible that glypican mediates different effects depending on its cellular source. Several heparin-binding proteins bind specifically to subfractions of heparin, suggesting that these proteins recognize aspects of GAG structure that may be found

only on some HS chains (Marcum and Rosenberg, 1989; Lee and Lander, 1991; Nurcombe et al., 1993; San Antonio et al., 1993). Purification of glypican from specific neural sources, followed by binding studies with heparin-binding proteins of the brain, should indicate whether heterogeneity in the HS borne by the glypican core protein influences the kinds of molecules for which glypican, in different types of neurons, may act as a receptor.

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