The Journal of Neuroscience, June 1992, 12(6): 2393–2402

Variable Membrane Glycoproteins in Different Growth Cone Populations

Ha-nan Li,[a] Santiago Quiroga, and Karl H. Pfenninger

Department of Cellular and Structural Biology, University of Colorado School of Medicine and University of Colorado Cancer Center, Denver, Colorado 80262

The question of whether growth cones generated by different neurons contain distinctive membrane glycoproteins was examined. Growth cone particles (GCPs) were isolated from specific regions of fetal or early postnatal brain, and their membrane proteins were analyzed by 2D gel electrophoresis and Western blotting, using WGA as a probe. These blots were compared to those generated by synaptosomes from adult brain. The patterns reveal a number of WGA-binding glycoproteins that are uniformly present in these subcellular fractions and others that are found in GCPs from selected brain regions only. The results indicate, therefore, substantial pattern diversity for the different, restricted growth cone populations. Some of the WGA-binding glycoproteins seen in GCPs disappear with increasing age and are absent from synaptosomes, while others seem to become more prominent.

One of the glycoprotein complexes present in all GCP and synaptosome fractions analyzed is gp93. It has an apparent molecular weight of 90–97 kDa and exhibits unusually high heterogeneity in GCPs from whole fetal brain. The gp93 complex covers a pI range from about 4.9 to about 6.4 and consists of at least 12 different species, probably isoelectric variants. In GCPs from different brain regions, the sets of gp93 species observed are different and characteristic. Neuraminidase digestion shifts the gp93 pattern to a more neutral pI but simplifies it only partially, indicating that variable sialic acid content explains the molecular diversity to some extent. Thus, gp93 is a glycoprotein complex whose members are expressed and/or posttranslationally processed differentially in different growth cone populations. Such a glycoprotein family may be involved in selective cell–cell recognition.

The nerve growth cone is the leading edge of the growing axon and thus plays a crucial role in pathfinding and recognition for the establishment of neuronal networks. Therefore, growth cone plasmalemma is likely to contain molecules involved in selective cell–cell and cell–substratum interactions. Indeed, studies performed in recent years have identified a number of cell and substrate adhesion molecules in the growth cone (for review, see, e.g., McKay et al., 1983; Rutishauser and Jessell, 1988; Doherty and Walsh, 1989; Anderson, 1990). There is also increasing evidence for the participation of oligosaccharide structures in the definition of neuronal surface specificity (e.g., Jessell et al., 1990). In a small but increasing number of cases, such as the lymphoid system, oligosaccharides and their lectin-like receptors are now established as determinants involved in recognition mechanisms (Gallatin et al., 1986; Lasky et al., 1989).

This laboratory reported several years ago that growth cones formed by different neuron types exhibit on their surfaces specific sets of lectin receptors, suggesting the presence of a "surface carbohydrate signature" that could be involved in cell–cell recognition (Pfenninger and Maylié-Pfenninger, 1981; Pfenninger et al., 1984). By now, such diversity of cell surface molecules, often involving carbohydrate, has been reported for a number of neuron populations and systems (e.g., Dodd et al., 1984; Levitt, 1984; Harrelson and Goodman, 1988; Bajt et al., 1990; Zaremba et al., 1990). In the meantime, membrane glycoproteins of growth cones isolated by subcellular fractionation [growth cone particles (GCPs)] were analyzed by 1D and 2D gel electrophoresis combined with lectin blotting (Greenberger and Pfenninger, 1986). These studies showed that GCPs from whole brain, that is, from many different neuron types, contain a very large number of minor glycoprotein species. This observation raised the question of whether each growth cone contains a highly heterogeneous glycoprotein population, or whether the pattern observed for whole brain is the composite of different sets of glycoproteins contributed by different growth cone types, as the lectin mapping studies suggest. The present report examines this question. A method has been developed for the scaled-down fractionation of GCPs from small brain regions, and such fractions have been characterized. Membrane glycoproteins of GCPs from specific brain regions are described by 2D gel electrophoresis and detected by lectin blotting, that is, on the basis of their oligosaccharide residues. The reported observations are confined to glycoproteins binding WGA because WGA (without oxidative/reductive phenylamination; cf. Greenberger and Pfenninger, 1986) produces a relatively simple but striking glycoprotein pattern for GCPs. WGA binds with high affinity and in a sialic acid–independent manner to internal *N*-acetyl glucosamine (GlcNAc) residues, especially those in hybrid N-linked oligosaccharides containing a bisecting GlcNAc and those in polylactosamine-type glycans containing the repeat Galβ(1→4)GlcNAcβ(1→3). WGA also binds to arrays of multiple external sialic acid residues, but with much lower affinity (Yamamoto et al., 1981; Gallagher et al., 1985). Under the

Received May 6, 1991; revised Jan. 17, 1992; accepted Jan. 21, 1992.

We are grateful to Dr. Vic Spitzer for his generous help with the computer analysis of the 2D gels, to Dr. Marie-France Maylié-Pfenninger for reading the manuscript, to Virginia Miller for assistance with electron microscopy and photography, and to Carmel McGuire for secretarial help. This work was supported by NIH Grant NS24676.

Correspondence should be addressed to Dr. Karl H. Pfenninger, Department of Cellular and Structural Biology, University of Colorado Health Sciences Center, 4200 East Ninth Avenue, B-111, Denver, CO 80262.

[a] Present address: Department of Anatomical Sciences and Neurobiology, University of Kentucky, Louisville, KY.

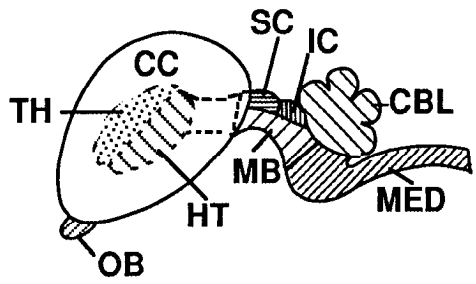

Figure 1. Schema of developing rat brain to illustrate the different brain regions dissected for the preparation of regional growth cone fractions. *CBL,* cerebellum; *CC,* cerebral cortex; *HT,* hypothalamus; *IC,* inferior colliculus; *MB,* midbrain; *MED,* medulla; *OB,* olfactory bulb; *SC,* superior colliculus; *TH,* thalamus. At F18, the superior and inferior colliculi were not dissected away from the midbrain.

conditions used for the experiments reported here, high-affinity binding sites are revealed almost exclusively. 1D and 2D blots have been probed with other lectins as well, but the results do not seem to add significantly to the main conclusions of this study. We report here on WGA-binding glycoproteins present in all GCPs versus others restricted to GCPs from specific brain regions. These glycoproteins include a complex with an average M_r of 93 kDa (gp93) that contains an unusually large number of species that differ for specific brain regions.

Materials and Methods

Materials. Bovine serum albumin (BSA), hemoglobin, *Clostridium perfringens* neuraminidase (type X) (protease-free), leupeptin, sucrose, *N*-Tris (hydroxylmethyl)methyl-2-aminoethane sulfonic acid (TES), Tris (as Trizma base), EDTA, pepstatin, urea, polyvinylpyrrolidone (average MW, 40,000), and *N*-acetylglucosamine were from Sigma Chemical Co. (St. Louis, MO). 4-Chloro-1-naphthol was from Aldrich Chemical Co. (Milwaukee, WI). Acrylamide, *N,N'*-methylene-bis-acrylamide, SDS, and Affigel *N*-acetylglucosamine were from Bio-Rad Laboratories (Richmond, CA). Ampholytes were from Serva Fine Biochemicals (New York, NY). Nitrocellulose was from Gelman Sciences (Ann Arbor, MI), and aprotinin (Trasylol), from Bayer Leverkusen, Germany (distributed by Miles, Inc., FBA Pharmaceuticals, West Haven, CT), $Na^{125}I$ was from ICN Radiochemicals Inc. (Irvine, CA). Anti-tubulin and anti-actin IgG were from Biomedical Technologies, Inc. (Stoughton, MA). Anti-rabbit IgG-peroxidase was from Boehringer Mannheim Biochemicals (Indianapolis, IN). All other chemicals were reagent grade and purchased from Sigma Chemical Co. or Fisher Scientific Co. (Pittsburgh, PA).

Subcellular fractionations. The experimental animal for these studies is the rat. GCPs were isolated from whole brain at fetal day 18 (F18) or postnatal day 8 (P8) according to published procedures (Pfenninger et al., 1983). Synaptosomes were isolated as described from adult rat cerebral cortex or selected other brain regions, after removal of white matter (Ellis et al., 1985).

For GCP fractionation from specific regions of developing brain (F18 or P8), the following method was developed. Dissected tissue samples pooled from about 12–24 fetal or postnatal brains were homogenized gently in about 10 vol of 0.32 M sucrose with 1000 U/ml Trasylol and 30 μM leupeptin added. The samples were spun for 10 min at 3000 rpm in a JS 7.5 rotor using a Beckman J2-21 centrifuge. The low-speed supernatant (0.75 ml) was layered onto a discontinuous sucrose density gradient consisting of 0.5 ml of 2.66 M sucrose and 0.75 ml of 0.80 M sucrose. All sucrose solutions contained 1 mM TES-NaOH (pH 7.3) and 1 mM $MgCl_2$. The gradients were spun at 75,000 rpm for 8 min (acceleration setting 5, deceleration setting 7) in a Beckman TL-100 microultracentrifuge, using a vertical rotor (TLV-100). The band at the sample/0.80 M sucrose interface was collected and frozen immediately to −80°C. For analysis, the samples were thawed by the addition of an equal amount of lysis buffer (6 mM Tris-HCl, pH 8.1; 0.5 mM EDTA) and kept for 40 min at 4°C prior to pelleting of the membranes and solubilization in electrophoresis buffer. Protein amounts in the different fractions were determined according to Lowry et al. (1951). The different brain regions subfractionated are illustrated in Figure 1. The dissection of large numbers of developing brains cannot be very accurate, so the designations are intended to indicate the general region rather than a precise anatomical area.

Neuraminidase digestion. Some GCP samples were digested with neuroaminidase from *Clostridium perfringens* prior to electrophoresis as follows. Samples of 100–200 μg membrane protein in 500 μl buffer were incubated for 6 hr with a total of 0.4 U neuraminidase at 37°C. The incubation buffer consisted of 0.1 M sodium acetate (pH 5.0), 2.0 mM $CaCl_2$, 0.2 mM EDTA, 100 U/ml Trasylol, 1 μg/ml pepstatin A, and 30 μM leupeptin. The incubation was terminated by the addition of solubilization buffer (O'Farrell, 1975) and solid urea to bring the final concentration to 9.0 M.

Polyacrylamide gel electrophoresis and Western blotting. Membranes collected by pelleting from lysed GCPs or synaptosomes were solubilized in sample buffer and their polypeptides resolved by two-dimensional gel electrophoresis (2D-PAGE), that is, isoelectric focusing followed by SDS-PAGE (O'Farrell, 1975). Equal aliquots of 200 μg protein were used for these gels. The separated polypeptides were electrotransferred onto nitrocellulose (Towbin et al., 1979; Greenberger and Pfenninger, 1986). The blots were stained with amido black and then quenched for 48 hr in phosphate-buffered saline (PBS) containing 2% polyvinylpyrrolidone, 0.5% BSA, and 0.5% hemoglobin. Some of the gels were not blotted but analyzed by silver staining according to Morrissey (1981).

The blots were probed with ^{125}I-labeled WGA. WGA was purified on a *N*-acetylglucosamine affinity column (Shaper et al., 1973) and radioiodinated with the chloramine T method (Hunter and Greenwood, 1962). Dialyzed ^{125}I-labeled WGA was repurified on the *N*-acetylglucosamine column and then redialyzed. The quenched blots were incubated with the iodinating lectin for 8 hr in fresh quench solution. The amount of WGA used was between 0.4 and 2 μg of WGA, corresponding to approximately 10^7 cpm, in 60 ml solution. At this low concentration, high-affinity binding sites are detected almost exclusively. Control experiments included the hapten sugar *N*-acetylglucosamine (0.25 M) in the labeling solution and were blank. The blots were washed 3 × 15 min in PBS, dried, and then sandwiched with x-ray film (Kodak X-Omat AR) for radioautography at −80°C.

Most of the Western blots were subsequently labeled with polyclonal antibodies against actin and β-tubulin. The blots were wetted and requenched in 0.5% hemoglobin in PBS for 2 hr. They were then incubated in rabbit anti-chicken tubulin and rabbit anti-bovine actin (diluted 1:500 in quenched solution) for 2 hr, washed 3 × 20 min, and then reacted with goat anti-rabbit IgG conjugated with peroxidase (1:500 in quench solution) for 2 hr. After three washes of 20 min each, horseradish peroxidase was detected using 4-chloro-1-naphthol as a chromogen (Hawkes et al., 1982).

In order to ascertain reproducibility of the results, 2D-PAGE and blotting were executed on at least two, but usually three or more independently collected samples for each brain region and time point. The internal standards, actin and tubulin, were used to monitor electrophoretic parameters. Because of the inevitable small pattern shifts seen in 2D-PAGE, subtle variations among the patterns are not described, and only those features and differences are discussed that are seen in all the blots.

For detailed gp93 analysis, radioautograms were digitized on a Xerox Datacopy GS plus attached to a Macintosh II computer using MACIMAGE version 2.30 software. The digitized images were normalized using the positions of actin and tubulin in the gels as a bias. These manipulations, as well as the densitometry shown in Figure 7, were done on the same computer using the IMAGE version 1.33 software. Relative densities of the normalized radioautograms were rendered in RGB pseudocolor with Adobe PHOTOSHOP software.

Electron microscopy. GCP samples resulting from the scaled-down fractionation procedure were also analyzed ultrastructurally. The samples were fixed and processed as described by Pfenninger et al. (1983). For stereologic analysis, two independently collected GCP fractions of P8 cerebellum were embedded in two blocks each for sectioning. A total of 60 electron micrographs were taken systematically across several sections of each of these blocks, at a primary magnification of 1,500×. Prints (enlarged approximately 2.5×) were overlaid with a 2 × 2 cm grid, and the points falling onto structures in the fraction were classified (Weibel and Bolender, 1973). A total of over 3000 points in five groups were tallied. In this analysis, the percentage of points on a specific structure is proportional to the volume fraction (V/V_0) of that structure, relative to the total volume of elements in the preparation. Structures unidentifiable due to an unfavorable plane of section ($V/V_0 = 0.34 \pm 0.08$) were deducted from the total.

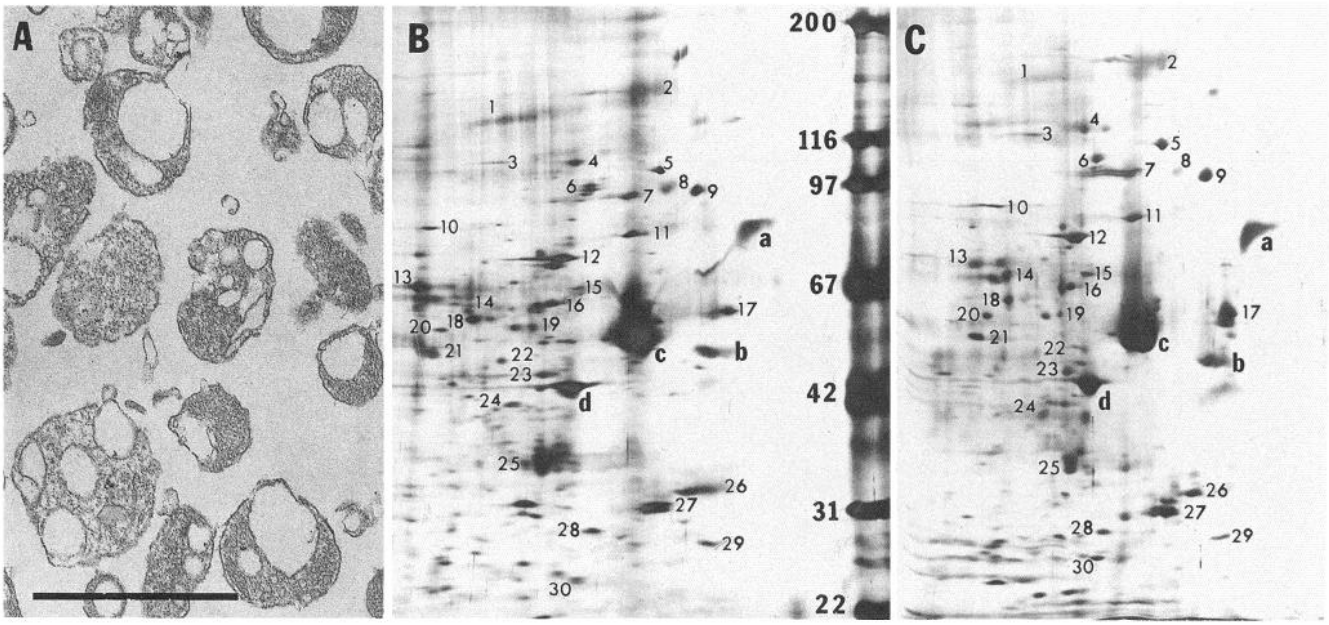

Figure 2. *A* is an electron micrograph of GCPs prepared from P8 cerebellum. This fraction is very similar morphologically to that prepared from whole brain and described previously (Pfenninger et al., 1983). Scale bar, 1 μm. *B* and *C* show silver-stained 2D gels of GCPs from P8 cerebellum (*B*; with standard lane) and from F18 whole brain (*C*): 25 μg protein were loaded on each gel. *a–d* identify known major polypeptides of GCPs (*a*, 80 kDa phosphoprotein; *b*, GAP43/pp46; *c*, tubulin; *d*, actin). Many of the corresponding minor polypeptide spots or complexes are *numbered* to facilitate comparison of the two gels. Differences are evident only in complexes *14* and *21* and below 31 kDa. Otherwise there is a high degree of similarity between the two polypeptide patterns. Molecular weight standards are indicated in kDa. The acidic end of the gels' pH gradients is on the *right*.

Results

GCPs isolated according to the scaled-down procedure are morphologically identical to those isolated by the standard procedure (Figure 2*A*). Compared to that from whole fetal brain, the GCP preparation from P8 cerebellum contains more numerous membrane "ghosts" ($V/V_0 = 0.33 \pm 0.21$), many of them most likely the result of GCP lysis. Nevertheless, the fraction is remarkably homogeneous. The total of identifiable non-GCP elements in the fraction is less than 0.08 (V/V_0). Most prominent among these are occasional myelin fragments ($V/V_0 = 0.067 \pm 0.046$), whereas synaptosomes or presynaptic endings ($V/V_0 = 0.0024 \pm 0.0029$) and other foreign elements ($V/V_0 = 0.0042 \pm 0.0051$) are very rare. GCPs are the most prominent structures in the fraction, at $V/V_0 = 0.60 \pm 0.24$. GCPs isolated from P8 cerebellum were compared biochemically to those isolated from F18 whole brain using 2D-PAGE and silver staining (Fig. 2*B,C*). In order to assist with the comparison, some of the major, characteristic spots have been labeled with letters (see caption). Many of the minor spots are numbered; with the exception of the complexes to the left of numbers 14 and 21 and of some of the spots below $M_r = 31$ kDa, the patterns are very similar. Digitizing and normalizing the gels for the positions of the major polypeptides, especially actin and tubulin, results in essentially superimposable patterns, with the exceptions noted above (data not shown). Therefore, GCPs isolated on a microscale can be considered equivalent to those isolated from whole brain.

General glycoprotein patterns

To establish a baseline for comparison, GCPs were isolated from whole brain at F18 and P8, and synaptosomes were fractionated from adult cerebral cortex. The three fractions were analyzed in terms of their WGA-binding glycoproteins. In these and all subsequent 2D-PAGE blots, the positions of β-tubulin (left, pI 5.2; Righetti and Tudor, 1981) and actin (right, pI 5.5; Righetti and Tudor, 1981) are indicated by dashes at the bottom of the radioautograms. Prominent glycoprotein complexes, comparable based on their mobilities in two dimensions, are numbered (for their apparent molecular weights, see Tables 1, 2). The full pH range of these gels is from about 4.1 to about 6.5. The resulting blots (Fig. 3*A–C*) illustrate WGA-binding patterns simpler than those obtained after enhancement with oxidative/reductive phenylamination (Greenberger and Pfenninger, 1986). The three patterns are clearly related but not identical. The glycoprotein complexes 1 and 2 present in GCPs at F18 diminish in size with increasing age, and complex 1 is only barely detectable in synaptosomes. In contrast, complex 3 is relatively minor in the "young" GCPs but increases in intensity with age, to reach a maximum level of labeling in synaptosomes. The most prominent WGA-binding region is a broad streak (arrowhead) consisting of numerous, tightly spaced species between about 90 and 97 kDa. This glycoprotein complex will be referred to as gp93. While the complex is prominent in all three preparations, the most intense labeling shifts from an acidic pI (approximately 4.9–5.4) in GCPs isolated at F18 to a more neutral pI in GCPs isolated at P8 and, especially, in synaptosomes.

Because of the minor contamination of GCP fractions isolated at P8 with myelin fragments, we assessed the contribution that myelin could be making to the WGA-binding glycoproteins. White matter was dissected from adult brain, homogenized, and spun at low speed as described for developing brain tissue. The low-speed supernatant was then loaded onto the sucrose gradient designed for GCP isolation. After centrifugation, the band at the sample/0.8 M sucrose interface (usually containing the GCPs) was removed from the gradient and analyzed by 2D-

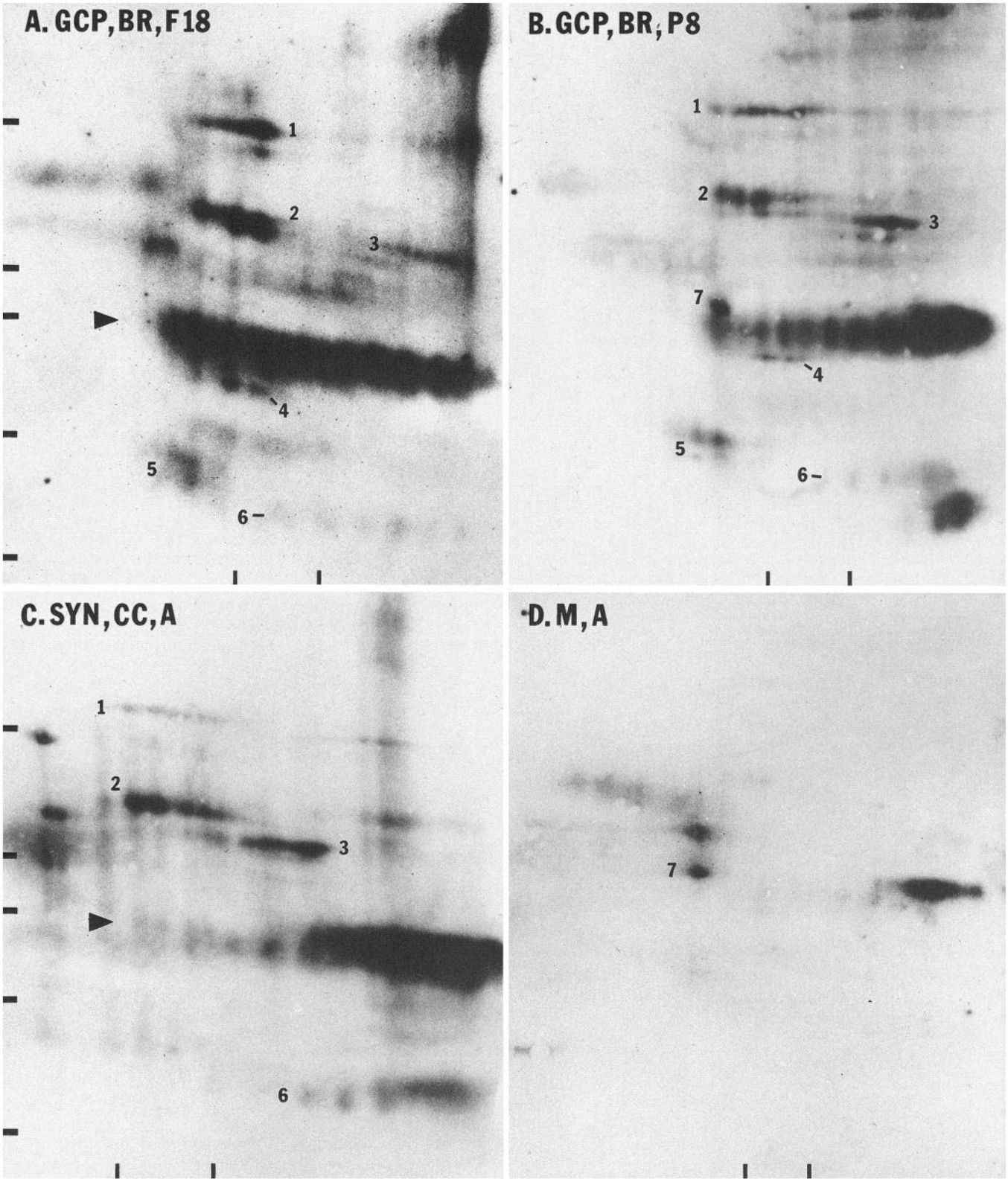

Figure 3. Radioautograms of WGA-labeled blots of 2D gels (equal amounts of protein loaded). *A* and *B* show the patterns for GCPs isolated from whole brain (*BR*) at F18 and P8, respectively. WGA-binding glycoproteins of synaptosomes (*SYN*) isolated from adult cerebral cortex (*CC*) are illustrated in *C*, and those of a "pseudo-myelin" fraction prepared from adult brain in *D*. Corresponding glycoprotein complexes are *numbered*. The *arrowheads* point to gp93. Molecular weight standards are indicated on the *left* (*from top to bottom*): 200, 116, 97, 67, and 42 kDa. *Dashes* at the bottom of each panel indicate the positions of β-tubulin (*left*, pI 5.2) and actin (*right*, pI 5.5) used as internal pI standards.

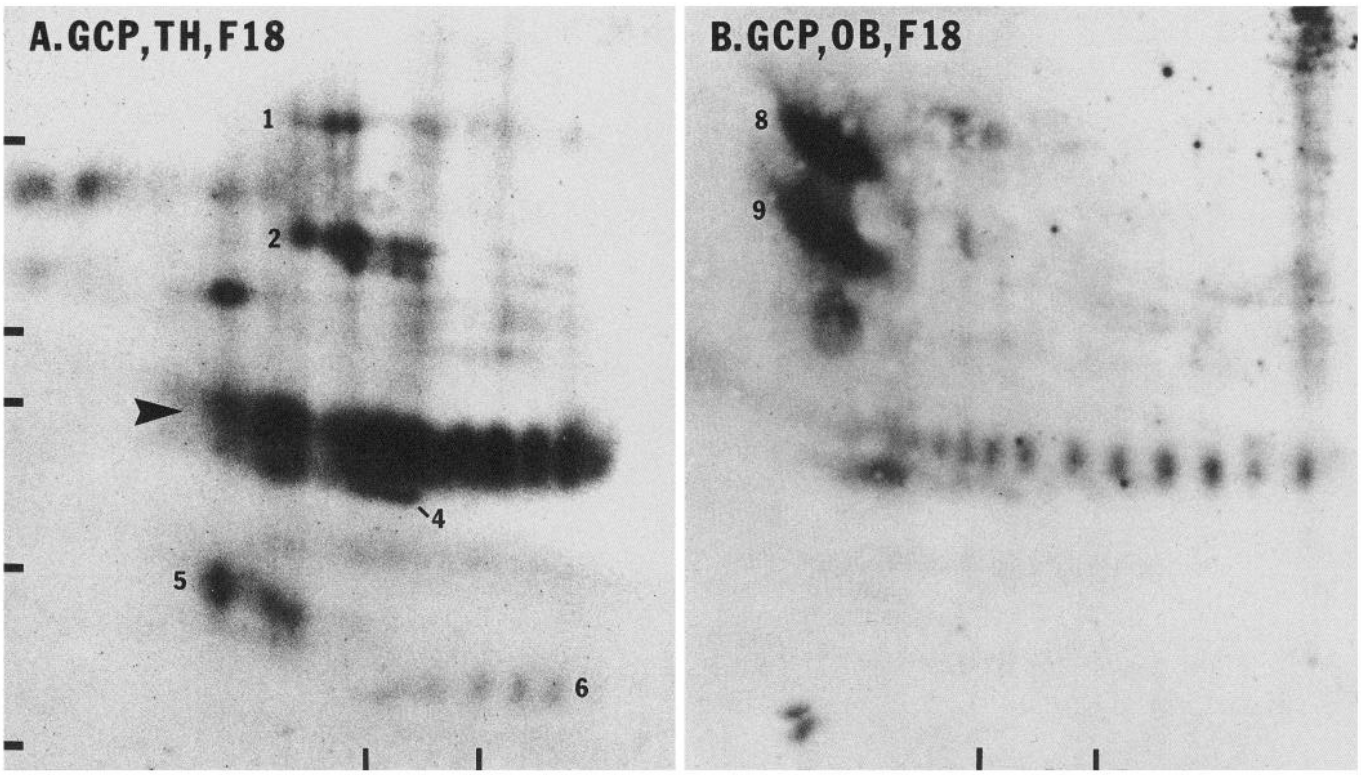

Figure 4. A and *B*, WGA-binding patterns of GCPs isolated at F18 from thalamus (*TH*) and olfactory bulb (*OB*), respectively (equal amounts of protein loaded). Conventions are as in Figure 3. The *arrowhead* points at the gp93 complex. For further description, see Results.

PAGE and WGA labeling. The result is shown in Figure 3*D*. There are only three major WGA-binding glycoprotein species in this preparation. These include a streak at neutrality that may be overlapping with the least acidic portion of gp93 and two additional, more acidic spots. The upper one of these (approximately 120 kDa) has not been seen in the other blots, whereas the lower one (complex 7) is detectable in several of the GCP preparations isolated at P8 (see Figs. 3*B*, 5*A,C,D*). The GCP fraction isolated at P8 from cerebellum, which is a late-developing structure, does not contain complex 7. The three WGA-binding glycoproteins detected in our "pseudo-myelin" preparation could be myelin derived or axon derived, but they do not significantly alter the GCP or synaptosome patterns reported here.

Regional patterns of GCP membrane glycoproteins

Figures 4 and 5 illustrate 2D patterns of WGA-binding glycoproteins of GCPs isolated at F18 and P8, respectively (refer to Fig. 1 for identification of the fractionated areas). The examples shown have been selected so as to facilitate the comparison of (1) GCPs from *different brain regions at the same age* as well as (2) GCPs from the same region at *different ages*. In Figure 4, thalamus (TH) and olfactory bulb (OB) GCPs, isolated at F18, are compared. gp93 is common to both patterns, but more prevalent in the thalamus. Furthermore, the arrays of isoelectric variants are different in the two cases. Other than that, the two patterns are rather different. Complexes 1 and 2 are not prominent in the olfactory bulb, which in turn contains complexes 8 and 9 that seem to be present only in this brain region. The label seen (Fig. 4*B*) in cerebellum (CBL) in a position similar to that of complex 8 in olfactory bulb (OB) is considerably more acidic and exhibits a different morphology, so it is probably not related to that glycoprotein.

At P8 (Fig. 5), the two glycoprotein patterns for thalamus and olfactory bulb, respectively, are quite similar to those just described, but most of the WGA labeling of gp93 is now shifted to a more neutral isoelectric point. gp93 labeling in the olfactory bulb has increased. For thalamus, complex 1 labeling is decreased significantly, but complex 3 has emerged in this tissue as well as in olfactory bulb. Complexes 5 and 6 are also detectable at P8 in both thalamus and olfactory bulb. The patterns shown for additional comparison, those of cerebellum and midbrain (MB), are again different, but related. Both contain prominent gp93 complexes, but different sets of species are seen. Midbrain contains complexes 1, 2, and 3, but lacks complexes 8 and 9. Cerebellum exhibits a weak complex 3, but except for gp93 none of the other glycoprotein species are clearly detectable. As already noted, complex 7 is present in thalamus, olfactory bulb, and midbrain at P8 but not in the late-developing cerebellum.

The presence and absence of some of these glycoprotein complexes are summarized in Tables 1 and 2. Overall, there are glycoproteins that are common to GCPs from all brain regions analyzed and others, such as complexes 8 and 9, that appear to be unique to a specific brain region. gp93 is unusual in that it is prominent in all brain regions, but its subspecies appear to be different regionally. This aspect is analyzed in further detail below. Table 1 also includes data from 2D-PAGE blots of synaptosomes isolated from specific brain regions. At this stage of differentiation, one also finds differences between the various glycoprotein patterns. These patterns are related to, but not the same as, those observed for GCPs isolated from the same regions.

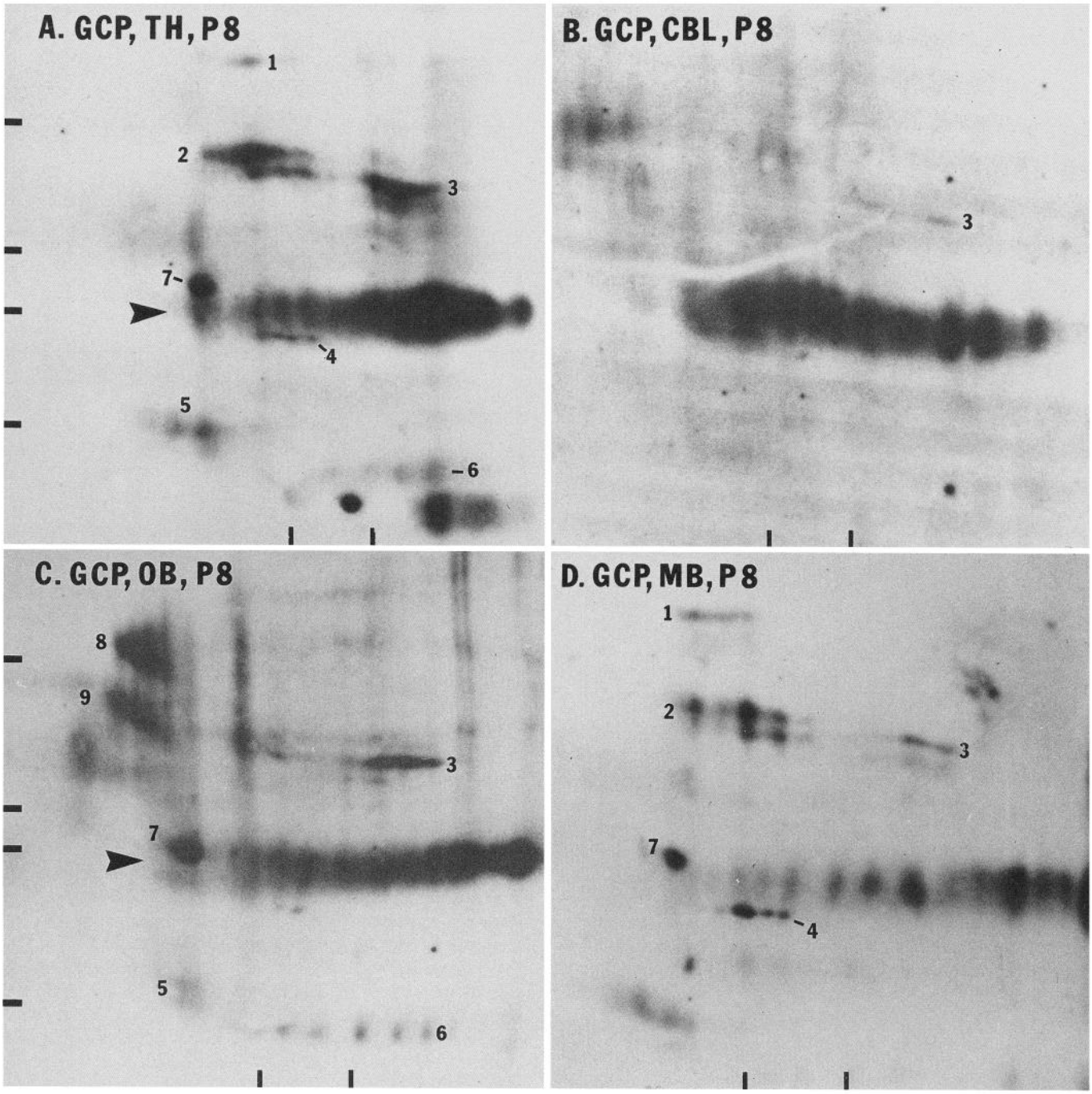

Figure 5. WGA-binding patterns of GCPs isolated at P8 from thalamus (*A*), cerebellum (*B*), olfactory bulb (*C*), and midbrain (*D*) (equal amounts of protein loaded). Molecular weight standards are (*from top to bottom*) 200, 116, 97, and 67 kDa. Other conventions are as in Figure 3. The *arrowheads* point at gp93 complexes. For further description, see Results.

Detailed analysis of gp93

Because of the difficulty of reproducing and calibrating 2D gels precisely (especially in the first dimension), we used the relative positions of endogenous actin and β-tubulin, revealed by immunoblotting, as internal standards. As described in Materials and Methods, the positions of these two proteins were transferred onto the radioautograms and the radioautographic patterns tethered in the two dimensions using the digitized image. This allows for the objective comparison of the arrays of gp93 species, as shown in Figure 6. Illustrated are gp93 patterns of GCPs from five different brain regions at F18 (*A*) and eight different brain regions at P8 (*B*). All gp93 patterns have been aligned according to isoelectric point, using the actin and tubulin markers (dashes at the bottom of the panels; β-tubulin, left; actin, right). There are several remarkable features of these patterns. They span a very wide pI range (from about 4.9 to about 6.4). They may consist of a very large number of species or variants. In some of these patterns, one can count at least 12 spots (e.g., MB at F18, HT at P8). The spacing between these spots seems to differ for different patterns. With increasing age, maximum labeling of the pattern shifts from a quite acidic pI

Table 1. Developmental changes in WGA-binding proteins

Complex (kDa)	Brain			CC			MB			OB		
	F18	P8	Syn	F18	P8	Syn	F18	P8	Syn	F18	P8	Syn
1 (204)	+	+	(+)	+	(+)	(+)	+	+	−	−	−	−
2 (150)	+	+	+	+	+	+	+	+	+	+	?	+
3 (134)	+	+	+	?	+	+	?	+	+	+	+	+
4 (83)	+	+	−	(+)	(+)	−	+	+	−	?	−	?
5 (65)	+	+	−	(+)	+	−	(+)	+	−	−	(+)	+
6 (54)	(+)	+	+	−	(+)	+	−	−	−	−	(+)	−
7 (100)	−	+	−	−	−	−	−	+	+	−	+	+
8 (196)	−	−	−	−	−	−	−	−	−	+	+	+
9 (156)	−	−	−	−	−	−	−	−	−	+	+	+
gp93	+	+	+	+	+	+	+	+	+	+	+	+

GCPs isolated at F18 or P8 are compared with synaptosomes (Syn; adult) from whole brain, cerebral cortex (CC), midbrain (MB), or olfactory bulb (OB). The glycoprotein complexes seen in Figures 3–5 are listed by complex number, and their M_rs are indicated in parentheses. Presence or absence of specific glycoproteins is indicated by plus or minus signs, respectively. Weak presence is shown as (+). Unclear results are indicated by question marks.

to a pI that is closer to neutrality. This is also reflected in the comparison of early- versus late-developing brain regions dissected at the same age. For example, the array for medulla, which matures early, is most heavily labeled near neutrality compared to the other patterns at F18. Similarly, midbrain, which develops relatively early, exhibits at P8 a shift to the right relative to cerebral cortex and especially cerebellum, which develop later.

The age-dependent shift of gp93 toward neutrality has been assessed also quantitatively, by performing densitometry across the pattern. This is shown for GCPs (F18 and P8) and synaptosomes (adult) isolated from midbrain (Fig. 7). The densitometric tracings do not resolve individual isoelectric variants, but they show the overall distribution. At F18, gp93 from GCPs shows a relatively even distribution of WGA label over the entire pH spectrum, whereas that from P8 GCPs is maximally labeled in a pI range from approximately 5.8–6.1. In synaptosomes, gp93 labeling forms a large peak between these pIs. The small, isolated peak seen at the acidic end of the spectrum is likely to be a glycoprotein not related to gp93.

Shifts in isoelectric point in glycoproteins can be caused by the presence of differing amounts of sialic acid. In order to examine this issue, GCP membranes isolated from whole brain at P8 were subjected to exhaustive neuraminidase digestion (6 hr) and compared with controls (Fig. 6*C*). Neuraminidase treatment does not seem to affect WGA binding but can be seen to shift the pattern significantly toward neutrality. However, many variants persist, even at quite acidic isoelectric points.

Discussion

The scaled-down fractionation procedure presented here renders it possible to isolate GCPs from specific brain regions of the fetal and newborn rat. Morphologically, these GCP preparations are very similar to those isolated from whole fetal brain, with the exception of small amounts of myelin contamination if postnatal animals are used. This contamination must be borne in mind but does not significantly affect the data presented here. Of the three WGA-binding glycoproteins detected in 2D blots of the "pseudo-myelin" fraction, only one is seen in postnatal GCP preparations. Although this glycoprotein (complex 7) could be derived from myelin, it could also be a developmentally regulated axolemmal component. Overall, the regional GCP preparations offer the same advantages as those from whole brain, and they allow one to analyze restricted growth cone

Table 2. Regional differences in GCP glycoproteins at P8

Complex (kDa)	CC	MB	OB	MED	TH	CBL	HT	IC	SC
1 (204)	(+)	+	−	+	(+)	−	−	+	+
2 (150)	+	+	?	+	+	−	+	+	+
3 (134)	+	+	+	+	+	(+)	+	+	+
4 (83)	(+)	+	−	?	+	−	(+)	+	+
5 (65)	+	+	(+)	+	+	−	+	+	+
6 (54)	(+)	−	(+)	+	+	−	−	+	−
7 (100)	−	+	+	+	+	−	?	+	+
8 (196)	−	−	+	−	−	−[a]	−	−	−
9 (156)	−	−	+	−	−	−	−	−	−
gp93	+	+	+	+	+	+	+	+	+

The presence or absence of glycoprotein complexes in GCP membranes isolated at P8 is shown for different brain regions (CC, cerebral cortex; MB, midbrain; OB, olfactory bulb; MED, medulla; TH, thalamus; CBL, cerebellum; HT, hypothalamus; IC and SC, inferior and superior colliculi, respectively). Symbols are as in Table 1.

[a] The labeled glycoprotein of about 200 kDa in P8 CBL is probably not identical to complex 8 because it is considerably more acidic.

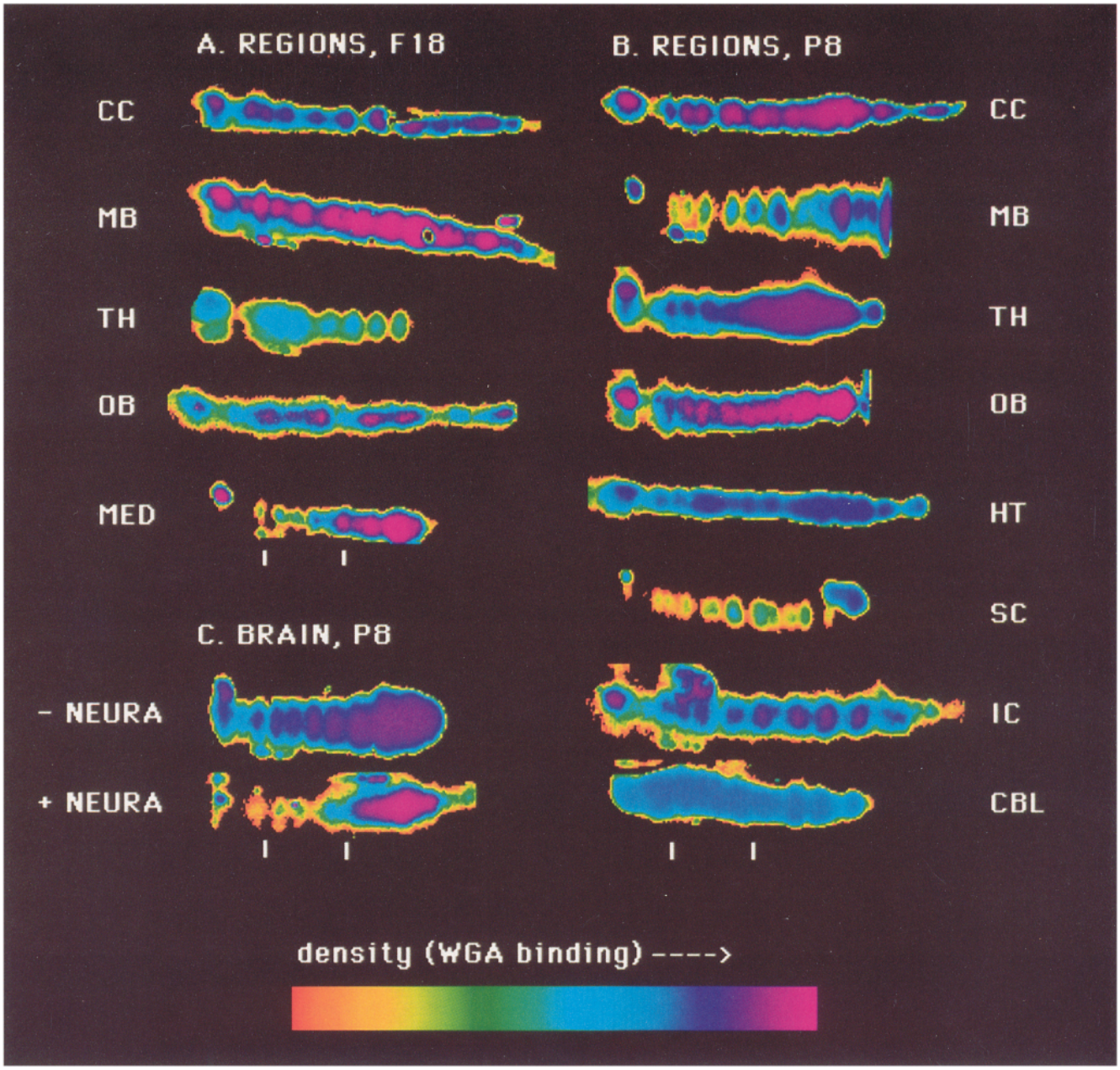

Figure 6. Pseudocolor rendering of WGA binding to gp93 complexes from different brain regions (*A* and *B*), and from whole brain with or without neuraminidase (±*neura*) digestion (*C*). The radioautographic patterns were digitized and normalized according to the positions of endogenous β-tubulin and actin (*vertical dashes*) as described in Materials and Methods. Note the large diversity of gp93 subspecies that differs from different brain regions, the shift to a more neutral pI with increasing age or maturity of the brain region, and the clear-cut but incomplete shift toward neutrality induced by neuraminidase treatment. For further description, see Results. For abbreviations, see Figure 1.

populations. In the case of the cerebellum, for example, the GCPs prepared at P8 are likely to consist mostly of parallel fiber growth cones because there are no other major fiber systems sprouting at this time in this structure.

The interpretation of the results presented here must take into account that even though the GCPs analyzed are of far less heterogeneous origin then those isolated from whole brain, one is examining growth cone *populations*. Furthermore, for the different regions dissected the degree of diversity of these populations is likely to differ. Therefore, the 2D blots shown are superimposition images that enhance the features common to the majority of GCPs contained in a preparation. Several age-dependent differences have been found in GCP fractions. Theoretically, these could be due to, for example, increasing amounts of synaptosome contamination. However, morphometric analysis shows that this is not a problem. Alternately, there may be shifts in the growth cone population recovered and/or there may be true maturation-dependent changes in growth cone membranes. Such changes could be caused, for example, by the formation of synapses *en passant* while the axonal tip continues to grow, whereby the formation of such synapses may lead to the expression of new glycoproteins throughout the axon.

Constant and variable glycoproteins of GCP membranes

As evident from the examples illustrated and summarized in Tables 1 and 2, the patterns of WGA-binding glycoproteins of GCP membranes are clearly related to one another but not identical. Some glycoproteins, such as complexes 2 and 3 as well as gp93, appear to be present in most or all GCP populations analyzed, whereas others, such as complexes 8 and 9, appear to be highly restricted in their distribution. The interpretation of these results has to take into account, however, that an oligosaccharide-specific probe (WGA) was used for detection of the glycoproteins. Thus, the absence of a particular glycoprotein spot in a blot means only that a *WGA-binding form* of this glycoprotein is missing. This glycoprotein's core polypeptide could be present in differently glycosylated and thus undetectable form.

Most WGA-binding glycoproteins are shared by some of the GCP populations but not others. The "constant" (generally shared) glycoproteins could be membrane components essential for growth cone function, such as widely distributed cell adhesion molecules, membrane transporters, and so on. The "variable," that is, partially shared, glycoproteins could be common to several different growth cone types, but their appearance in different GCP preparations could also be caused by the overlap of growth cone populations. The present analysis cannot distinguish between these possibilities. The only glycoproteins that appear to be unique to a specific GCP population are complexes 8 and 9, which we have detected in the olfactory bulb only. Without more detailed information it is impossible to know the identity of these proteins, but they are of considerable interest. An unusual glycoprotein pattern is that of cerebellar GCPs. Some of the generally present WGA-binding glycoprotein complexes are only barely detectable in, or are absent from, this preparation. The only exception is gp93, the most prominent complex by far. Whether this unusually simple WGA pattern is related to the putative high degree of homogeneity of these GCPs (mostly from parallel fibers) is not known. It should be noted, however, that lectins with different affinities, such as *Ricinus communis* agglutinin II (after neuraminidase digestion) and concanavalin A, reveal additional glycoproteins (data not shown) not visible in the WGA radioautograms presented here.

Some maturation-dependent changes are observed in these patterns. For example, complex 1 becomes very faint or disappears as one compares GCPs from F18 brains with those from P8 brains and with synaptosomes. Similarly, complex 4 is present in GCPs but not in synaptosomes. At the same time, complex 3 becomes more intensely labeled with increasing age and in synaptosomes, suggesting that it is related to the synapse. As already discussed, such a change could be caused by a shift in the GCP populations or, more likely, by biochemical changes in the GCP membranes.

The heterogeneous membrane glycoprotein gp93

As already mentioned, gp93 is one of the uniformly present WGA-binding proteins in GCPs and synaptosomes, but it is unique in that it consists of an unusually heterogeneous family of subspecies. The gp93 complex consists in some cases of at least 12 individual WGA-binding glycoprotein spots, covering a wide pI range from about 4.9 to 6.4. Additional species may be uncovered by other lectins. Nevertheless, the complex migrates in 2D-PAGE as a continuous array of spots that lie within a molecular weight range of 90–97 kDa. Such a pattern is characteristic of a set of isoelectric variants of a single glycoprotein. This possibility was tested, in part, by subjecting GCPs to exhaustive neuraminidase digestion prior to analysis. As expected for glycoprotein species carrying differing amounts of sialic acid, this treatment deletes some of the spots and shifts the pattern toward a more neutral pH. However, a considerable amount of heterogeneity persists so that differential sialylation is unlikely to account for the full spectrum of diversity. Interestingly, WGA labeling is resistent to neuraminidase digestion, an observation that is explained by the structure of its high-affinity binding sites (see introductory remarks). The age-dependent shift of the pattern toward a more neutral pH also is consistent with a role of sialic acid in the generation of diversity; it is reminiscent of a similar shift observed for N-CAM as it loses its polysialic acid during brain maturation (Rothbard et al., 1982; Rougon et al., 1982). It should be noted, however, that the pI variance observed for 5B4-CAM, a member of the N-CAM family, covers a range of less that 0.5 pH unit (Ellis et al., 1985) and thus is much narrower than that for gp93. This raises the question of what other biochemical differences may account for the high diversity of gp93. The most likely candidates are sulfation, which could be present in the carbohydrate portion(s) of the molecule, or phosphorylation, which would be located most likely on a putative cytoplasmic tail of the glycoprotein. A further possibility, given the high degree of diversity, is that the gp93 complex consists of a glycoprotein family whose members are distinguished, in part, by their amino acid sequences. In other words, a more detailed analysis of this glycoprotein complex will have to consider not only differences in posttranslational modification, especially glycosylation, but also the presence of a variable primary sequence domain. These questions are being investigated at the present. gp93 could possibly be known already from other studies, but based on its molecular weight, there is no obvious match with the glycoproteins that have been described (see, e.g., Anderson, 1990). Certainly the molecule's high degree of diversity has not been observed before in growth cone membrane proteins.

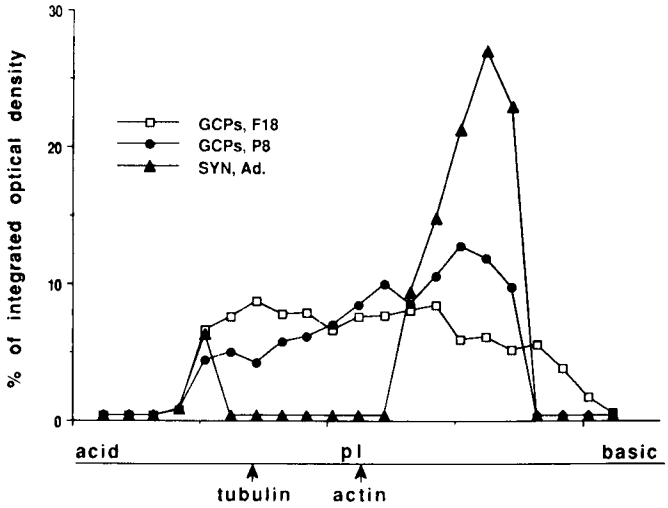

Figure 7. Densitometric analysis of the gp93 complex in GCPs from F18 and P8 animals and in synaptosomes (adult), all isolated from midbrain. The spatial resolution is approximately 5.5 mm in the original gel, corresponding roughly to the width of an average gp93 subspecies. Thus, the densitograms do not resolve the individual subspecies but show the relative amounts of WGA labeling across the pI range of the complex. For further description, see Results.

Overall, the regionally different patterns of WGA-binding glycoproteins in growth cones indicate that much of the glycoprotein diversity seen in large growth cone populations is due to the contribution of different, specific glycoprotein sets on individual growth cones, as originally proposed. Our observations indicate also that growth cones formed by different neuron types contain systematically different sets of gp93 subspecies, and/or that different neuron types process gp93 posttranslationally in a systematically different manner. Thus, this glycoprotein complex may be an excellent candidate for a (family of) recognition molecule(s), conveying upon the growth cone surface a highly specific identity for intercellular interactions, at least in terms of the exposed oligosaccharides.

References

Anderson H (1990) Adhesion molecules and animal development. Experientia 46:2–13.

Bajt ML, Cole RN, Zipser B (1990) The specificity of 130-kDa leech sensory afferent proteins is encoded by their carbohydrate epitopes. J Neurochem 55:2117–2125.

Dodd J, Solter D, Jessell TM (1984) Monoclonal antibodies against carbohydrate differentiation antigens identify subsets of primary sensory neurones. Nature 311:469–472.

Doherty P, Walsh FS (1989) Neurite guidance molecules. Curr Opin Cell Biol 1:1102–1106.

Ellis L, Wallis I, Abreu E, Pfenninger KH (1985) Nerve growth cones isolated from fetal rat brain: IV. Preparation of a membrane subfraction and identification of a growth-dependent membrane glycoprotein expressed on sprouting neurons. J Cell Biol 101:1977–1989.

Gallagher JT, Morris A, Dexter TM (1985) Identification of two binding sites for wheat-germ agglutinin on polylactosamine-type oligosaccharides. Biochem J 231:115–122.

Gallatin M, St John TP, Siegelman M, Reichert R, Butcher EC, Weissman IL (1986) Lymphocyte homing receptors. Cell 44:673–680.

Greenberger LM, Pfenninger KH (1986) Membrane glycoproteins of the nerve growth cone: diversity and growth-associated oligosaccharides. J Cell Biol 103:1369–1382.

Harrelson AL, Goodman CS (1988) Growth cone guidance in insects: fasciclin II is a member of the immunoglobulin superfamily. Science 242:700–708.

Hawkes R, Niday E, Gordon J (1982) A dot-immunobinding assay for monoclonal and other antibodies. Anal Biochem 119:142–147.

Hunter WM, Greenwood FC (1962) Preparation of iodine-131 labelled human growth hormone of high specific activity. Nature 194:495–496.

Jessell TM, Hynes MA, Dodd J (1990) Carbohydrates and carbohydrate-binding proteins in the nervous system. Annu Rev Neurosci 13:227–255.

Lasky L, Singer MS, Yednock TA, Dowbenko D, Fennie C, Rodriguez H, Nguyen T, Stachel S, Rosen S (1989) Cloning of a lymphocyte homing receptor reveals a lectin domain. Cell 56:1045–1055.

Levitt P (1984) A monoclonal antibody to limbic system neurons. Science 223:299–301.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275.

McKay RDG, Hockfield S, Johansen J, Thompson I, Frederiksen K (1983) Surface molecules identify groups of growing axons. Science 222:788–792.

Morrissey JH (1981) Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Anal Biochem 117:307–310.

O'Farrell PZ (1975) High-resolution two-dimensional electrophoresis of proteins. J Biol Chem 250:4007–4021.

Pfenninger KH, Maylié-Pfenninger M-F (1981) Lectin labeling of sprouting neurons. I. Regional distribution of surface glycoconjugates during plasmalemmal expansion. J Cell Biol 89:536–546.

Pfenninger KH, Ellis L, Johnson MP, Friedman LB, Somlo S (1983) Nerve growth cones isolated from fetal rat brain. I. Subcellular fractionation and characterization. Cell 35:573–584.

Pfenninger KH, Maylié-Pfenninger M-F, Friedman LB, Simkowitz P (1984) Lectin labeling of sprouting neurons. III. Type-specific glycoconjugates on growth cones of different origin. Dev Biol 106:97–108.

Righetti PG, Tudor G (1981) Isoelectric points and molecular weights of proteins—a new table. J Chromatogr 220:115–194.

Rothbard JB, Brackenbury R, Cunningham BA, Edelman GM (1982) Differences in the carbohydrate structures of neural cell-adhesion molecules from adult and embryonic chicken brains. J Biol Chem 257: 11064–11069.

Rougon G, Deagostini-Bazin H, Hirn M, Goridis C (1982) Tissue- and developmental stage-specific forms of a neural cell surface antigen linked to differences in glycosylation of a common polypeptide. EMBO J 1:1239–1244.

Rutishauser U, Jessell TM (1988) Cell adhesion molecules in vertebrate neural development. Physiol Rev 68:819–857.

Shaper JH, Barker R, Hill RL (1973) Purification of wheat-germ agglutinin by affinity chromatography. Anal Biochem 53:564–570.

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354.

Weibel ER, Bolender RP (1973) Stereological techniques for electron microscopic morphometry. In: Principles and techniques of electron microscopy, Vol 3, Biological applications (Hayat MA, ed), pp 239–291. New York: Van Nostrand-Reinhold.

Yamamoto KT, Tsuji T, Matsumoto I, Osawa T (1981) Structural requirements for the binding of oligosaccharides and glycopeptides to immobilized wheat germ agglutinin. Biochemistry 20:5894–5899.

Zaremba S, Naegele JR, Barnstable CJ, Hockfield S (1990) Neuronal subsets express multiple high-molecular-weight cell-surface glycoconjugates defined by monoclonal antibodies Cat-301 and VC1.1. J Neurosci 10:2985–2995.