Glycosaminoglycans of Brain during Development†

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ABSTRACT: The concentration of hyaluronic acid, chondroitin sulfate, and heparan sulfate was measured in rat brain at 2-day intervals from birth to 1 month of age, and in 40-day-old and adult animals. The levels of all three glycosaminoglycans increased after birth to reach a peak at 7 days after which they declined steadily, attaining by 30 days concentrations within 10% of those present in adult brain. The greatest change was seen in hyaluronic acid, which decreased by 50% in 3 days, and declined to adult levels (28% of the peak concentration) by 18 days of age. Only heparan sulfate showed a significant change in metabolic activity during development (a fourfold increase in the relative specific activity of glucosamine), most of which occurred after 1 week of age. In 7-day-old rats almost 90% of the hyaluronic acid in brain is extractable by water alone, as compared to only 15% in adult animals, and this large amount of soluble hyaluronic acid in young rat brain is relatively inactive metabolically. On the basis of our data we propose that the higher amounts of hyaluronic acid found in very young brain may be responsible for the higher water content of brain at these ages, and that the hydrated hyaluronic acid serves as a matrix through which neuronal migration and differentiation may take place during early brain development.

Several reports have described the glycosaminoglycan (mucopolysaccharide) composition of rat (Singh and Bachhawat, 1965), human (Singh and Bachhawat, 1968), and feline (Young and Custod, 1972) brain at various stages of postnatal development. The data from these studies agree only in part, although postnatal human brain was analyzed at only three ages (neonatal, 1 year, and adult). Moreover, the most complete age study, that of rat brain, was performed before the glycosaminoglycan composition of brain was well established, and without the benefit of newer analytical methods which afford greater specificity and sensitivity. In the course of a more comprehensive investigation of glycosaminoglycans and glycoproteins during brain development, we have therefore studied the content and metabolism of glycosaminoglycans at various stages of the postnatal development of rat brain.

Experimental Section

Glycosaminoglycans were isolated and quantitated by methods described previously (Margolis and Margolis, 1970, 1972). These involve extraction of lipids with chloroform–methanol, digestion of the lipid-free protein residue with Pronase, dialysis, and precipitation of the glycosaminoglycans with cetylpyridinium chloride (CPC).[1] Glycopeptides derived from brain glycoproteins remain in the CPC supernatant, and are recovered after removal of excess CPC by precipitation with KSCN followed by dialysis. The sulfated glycosaminoglycans were separated from hyaluronic acid by differential precipitation with CPC from 0.3 M NaCl, and excess CPC was removed from the hyaluronic acid as described above for the glycopeptides.

The concentration of hyaluronic acid was determined from the glucosamine content of the supernatant obtained after precipitation of the sulfated glycosaminoglycans from 0.3 M NaCl, and heparan sulfate and chondroitin sulfate concentrations were based on the glucosamine and galactosamine content of the sulfated glycosaminoglycan fraction. Glycosaminoglycans were hydrolyzed for 3 hr at 100° in 6 N HCl, and glucosamine and galactosamine were determined using the amino acid analyzer.

For labeling studies, rats were injected intraperitoneally with [6-^{3}H]glucosamine (1 μCi/g body weight) and sacrificed after 6 hr. To investigate the relative percentages and specific activities of glycosaminoglycans extractable by various solvents in 7-day-old rat brain, 23 g of pooled brain were homogenized in 9 vol of cold distilled water and centrifuged for 1 hr at 100,000 *g* as described previously (Margolis and Margolis, 1973a,b). The pellet was then reextracted with 1% Triton X-100 (6 ml/g brain) and again centrifuged as described above to obtain a Triton-soluble fraction and an unextractable residue. A thin layer of material overlying the Triton pellet and with an appearance distinct from it was analyzed separately. It contained only 3.5% of the total glycosaminoglycan, and because the specific activities of its components were very similar to those found in the Triton extract, we have added this material to the Triton extract in calculating the distribution of glycosaminoglycans in the various fractions.

The specific activity of hyaluronic acid was determined directly from aliquots used for counting of radioactivity and quantitation of glucosamine. To determine the specific activity of heparan sulfate and chondroitin sulfate, the sulfated glycosaminoglycan fraction was digested with chondroitinase ABC, and the resulting chondroitin sulfate disaccharides were separated from the undegraded heparan sulfate by gel filtration on Sephadex G-25 (Margolis and Margolis, 1972, 1973b). Similarly, to determine the specific activity of hexosamine and sialic acid in the glycoproteins, glycopeptides were desialylated by mild acid hydrolysis (0.1 N H_2SO_4, 1 hr, 80°) and free sialic acid was separated from the desialylated glycopeptides by gel filtration on Sephadex G-15. This procedure yields radiochemically pure fractions of hexosamine-labeled glycopeptides and sialic acid (Margolis and Margolis, 1973b). Sialic acid was determined by the periodate–resorcinol method of Jourdian *et al.* (1971),

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[1] Abbreviation used is: CPC, cetylpyridinium chloride.

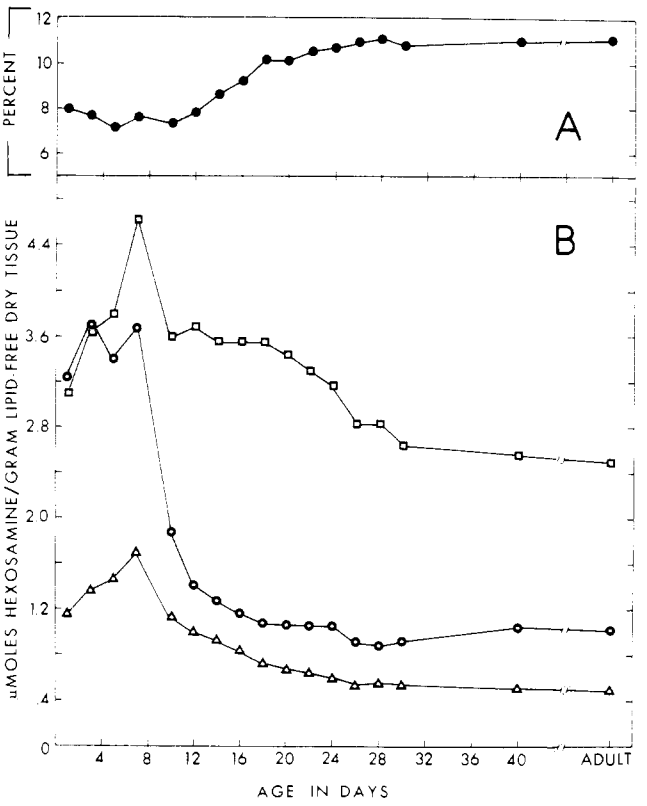

FIGURE 1: (A) Lipid-free dry weight as per cent of fresh weight of rat brain during postnatal development. (B) Concentration of glycosaminoglycans, expressed as micromoles of the constituent hexosamine per gram of lipid-free dry weight, as a function of increasing postnatal age. (□) Chondroitin sulfate; (○) hyaluronic acid; (△) heparan sulfate. For each age point 3–12 pooled brains were analyzed, and the 1- to 12-day age points represent the average values from two separate experiments (which gave essentially identical results).

and hexosamine was quantitated using the amino acid analyzer after hydrolysis of the glycopeptides for 8 hr at 100° in 4 N HCl.

Results

The concentration of all three rat brain glycosaminoglycans increases after birth to reach a peak value of 10 μmol of hexosamine/g of lipid-free dry weight at 7 days (Figure 1B). The postnatal increase in hyaluronic acid is relatively small, but after 7 days the concentration of this glycosaminoglycan declines precipitously to 51% of the peak level by 10 days, and reaches adult values by 18 days. Since the lipid-free dry weight of brain also increases during development, from *ca.* 7% (at 5 days) to 11% of the wet weight (Figure 1A), the changes in glycosaminoglycan concentration would be slightly less marked if expressed on the basis of fresh brain weight. Although at 18 days the level of chondroitin sulfate is still decreasing, the concentrations of both hyaluronic acid and heparan sulfate are very similar at this time to those found in adult brain, and by 30 days the levels of all three glycosaminoglycans are within 10% of their adult values.

It was also of interest to determine whether there were corresponding changes in the metabolic turnover of these glycosaminoglycans during brain development, as opposed to net changes in their levels due to biosynthesis or degradation. In the absence of steady-state conditions it is not possible to perform the usual type of *in vivo* turnover study, in which the decrease in specific activity of a product is followed for a period of time after labeling with a pulse of precursor. Nor can one simply compare the specific activities of the various products at different ages after a short period of labeling, since the resultant specific activities could be affected by factors other than age-related changes in turnover (*e.g.*, penetration of precursor into brain, or changes in the size of the endogenous precursor pool during development). We therefore chose to measure the relative specific activities of five different products synthesized from UDP-*N*-acetylhexosamine labeled by administration of [6-^{3}H]glucosamine. It is assumed in these studies that glucosamine is incorporated into hyaluronic acid, heparan sulfate, and the glycoproteins from a single pool of UDP-*N*-acetylglucosamine, and that the small pools of UDP-*N*-acetylglucosamine and -galactosamine are in isotopic equilibrium *via* the 4′-epimerase. Since developmental changes in the turnover of all five products in the same direction and to the same extent were considered possible but unlikely, it was hoped that this experimental design would reveal *relative* alterations in the turnover of one or more of the complex carbohydrates studied.

In fact, it was found that only heparan sulfate showed a marked change in metabolic activity during development, with a fourfold increase in relative specific activity, most of which occurred after 1 week of age (Table I). There was also a smaller increase in the relative specific activity of sialic acid in the glycoproteins between 1 and 7 days, and in the turnover of glycoprotein hexosamine during development to adulthood.

We have previously reported that almost two-thirds of the chondroitin sulfate in adult rat brain can be extracted by water alone, while only 15 and 22% of the hyaluronic

TABLE I: Incorporation of [^{3}H]Glucosamine into the Glycosaminoglycans and Glycoproteins of Rat Brain as a Function of Age.

	1 Day Old		7 Day Old		16 Day Old		Adult	
	Specific Activity[a]	RSA[b]	Specific Activity	RSA	Specific Activity	RSA	Specific Activity	RSA
Hyaluronic acid	31,500	1.0	15,600	1.0	17,200	1.0	8,000	1.0
Chondroitin sulfate	44,600	1.4	21,500	1.4	15,900	0.9	7,800	1.0
Heparan sulfate	69,000	2.2	43,600	2.8	78,300	4.6	71,900	9.0
Glycoprotein sialic acid	30,900	1.0	35,800	2.3	36,000	2.1	15,700	2.0
Glycoprotein hexosamine	87,000	2.8	60,200	3.9	76,400	4.5	37,500	4.7

[a] Cpm/μmol of hexosamine or sialic acid. Each sample consisted of pooled brains from 3 to 12 animals. [b] RSA = relative specific activity.

TABLE II: Extractability of Glycosaminoglycans in Young and Adult Rat Brain.[a]

	Hyaluronic Acid		Heparan Sulfate		Chondroitin Sulfate	
	7 Day Old (%)	Adult[b] (%)	7 Day Old (%)	Adult (%)	7 Day Old (%)	Adult (%)
Water extract	88	15	18	22	67	62
Triton X-100 extract	10	49	73	42	29	24
Residue	2	36	9	36	4	14

[a] Total of each column = 100%. [b] From Margolis and Margolis, 1973a.

TABLE III: Labeling of Glycosaminoglycans in Young and Adult Rat Brain.

	Hyaluronic acid			Heparan sulfate			Chondroitin sulfate		
	7 Day Old			7 Day Old			7 Day Old		
	Specific Activity[a]	RSA[b]	Adult RSA[c]	Specific Activity	RSA	Adult RSA	Specific Activity	RSA	Adult RSA
Water extract	10,100	0.4	2.2	37,600	1.0	0.5	15,900	1.1	1.1
Triton X-100 extract	53,600	2.0	2.4	25,100	0.7	0.7	21,200	1.4	1.4
Residue	26,600	1.0	1.0	37,500	1.0	1.0	15,100	1.0	1.0

[a] Specific activity expressed as cpm/μmol of hexosamine. [b] RSA = relative specific activity. [c] Calculated from data in Margolis and Margolis (1973b).

acid and heparan sulfate, respectively, are water extractable (Margolis and Margolis, 1973a). In view of our finding that the concentration of glycosaminoglycans, and especially of hyaluronic acid, is very high at 7 days of age, we also investigated whether there was a difference in the extractability of these compounds in young as compared to adult rat brain.

In 7-day-old rats almost 90% of the hyaluronic acid is water extractable (compared to 15% in adult brain), and only 2% is resistant to extraction with 1% Triton X-100 (compared to 36% in adult brain, Table II). Thus, the additional 2.6 μmol of hyaluronic acid glucosamine present per gram of lipid-free dry weight in 7-day-old as compared to adult rat brain can be more than accounted for by the increase in water-extractable hyaluronate (3.1 μmol/g). In the cases of heparan sulfate and chondroitin sulfate, the percentages of water-extractable material in young and adult rat brain were approximately the same, although much less of these glycosaminoglycans was resistant to extraction by Triton X-100 in young brain (Table II).

To determine whether the large amount of water-extractable hyaluronic acid in 7-day-old rat brain is actively involved in some developmental process requiring a high rate of renewal, in distinction to being present in a pool which is metabolically inert, we determined the relative specific activities of hyaluronate and of the other glycosaminoglycans separately in the water and Triton extracts and in the unextractable residue after 6 hr of labeling *in vivo* with [6-^{3}H]glucosamine. The results of this experiment are presented in Table III, together with the relative specific activities of the three glycosaminoglycans in corresponding fractions from adult rat brain, as calculated from previously published data (Margolis and Margolis, 1973b). It can be seen that the relative specific activity of water-extractable hyaluronic acid from young rat brain is only 20–40% as great as in the Triton extract and unextractable fractions, while in adult rat brain the relative specific activity of the soluble hyaluronate is the same or twice as high as that of the Triton-extractable or unextractable material (Table III). The relative specific activities of heparan sulfate and chondroitin sulfate are identical in each of the three fractions obtained from young and adult brain, with the exception of the water-extractable heparan sulfate which has a higher relative specific activity at 7 days than in the adult.

Discussion

There is general agreement among previous studies (Singh and Bachhawat, 1965, 1968; Young and Custod, 1972) that the glycosaminoglycan concentration of brain decreases with increasing age from the neonatal to the adult, and that this decrease is most striking in the case of hyaluronic acid. Our results agree with those of Singh and Bachhawat (1965) for rat brain and those of Young and Custod (1972) for feline brain in that the peak concentrations of both hyaluronic acid and chondroitin sulfate occur at approximately 1 week of age. However, we did not observe the decrease in concentration of these two glycosaminoglycans between 1 and 5 days as reported by Singh and Bachhawat (1965).

With the exception of heparan sulfate, there do not appear to be large changes in metabolic activity of the glycosaminoglycans during brain development. However, the relatively more rapid turnover of heparan sulfate after 1 week of age may indicate a role for this compound in brain functions which do not develop fully until this time, such as in the binding, storage, and release of neurotransmitter amines at nerve endings. We have previously presented data suggesting that chondroitin sulfate and heparan sulfate may be involved in such processes (Margolis and Margolis, 1973c; Margolis *et al.*, 1973).

In contrast to the situation in adult brain, almost all of the hyaluronic acid in young brain is readily extracted by water alone, and this large amount of soluble hyaluronic acid present until 8 to 10 days after birth has a relatively low metabolic activity. Polansky *et al.* (1974) have recently reported the presence of high levels of both hyaluronic acid and hyaluronidase activity in embryonic chick brain, and that these decrease rapidly after hatching. By analogy to other systems such as the regenerating newt limb, and the developing chick cornea, limb bud, and axial skeleton, these authors propose that hyaluronate inhibits certain cell interactions necessary for tissue formation, and that enzymatic or hormonal influences, or both, release this inhibition at the appropriate time.

In a previous study of brain hyaluronidase (Margolis *et al.*, 1972), hyaluronidase activity was assayed at 2-day intervals in rats from birth to 1 month of age, and at 1- to 3-week intervals from 1 month of age to adulthood. Although no significant changes in the enzyme activity in brain as a function of age were observed, if based on protein content rather than fresh weight of brain, the activity of hyaluronidase was 38% greater at 7 days and 68% greater at 10 days when compared to the levels in adult brain (unpublished results). However, these changes in hyaluronidase activity of neonatal rat brain are still much smaller than the three- to fourfold higher levels found in chick brain before as compared to after hatching (Polansky *et al.*, 1974). At this time most or all of the neuronal migrations have stopped in the chick (Hanaway, 1967), while they continue after birth in mammalian brain (Rakic, 1971). Our finding that hyaluronate concentrations do not begin to decrease in rat brain until 1 week after birth is therefore consistent with this different time-course of development of rat and chick brain, although we found no evidence that significantly higher levels of hyaluronidase are present in the very young brain. Indeed, higher levels of enzyme activity would not appear to be necessary, since we found that in both young and adult brain the hyaluronidase activity is 100- to 1000-fold greater than that required for the turnover rates of hyaluronic acid and chondroitin sulfate observed *in vivo* (Margolis *et al.*, 1972; Margolis and Margolis, 1973b).

It appears plausible that the high concentrations of soluble hyaluronic acid in very young brain may function in the retention of water, and thereby form a readily penetrable matrix through which neuronal migration and differentiation may take place during brain development. During the period between 1 and 3 weeks of age when the concentration of hyaluronic acid decreases by over 60% (from 116 μg/g wet wt of brain at 7 days to 43 μg/g at 22 days), the water content of rat brain decreases from 88 to 82% (De Souza and Dobbing, 1971).

The long polymeric chains of hyaluronic acid, with a molecular weight of 140,000 in brain (Margolis, 1967), are known to have a significant role in hydration and solvent transfer in tissues (Laurent *et al.*, 1969). Although there is no evidence for any extensive chemical binding of water to the polysaccharide chains, water is nonetheless immobilized in the solvent domain, which is greater than 10^3 times larger than the volume of the polysaccharide chain itself (Ogston and Stanier, 1951, 1953). Based on a hydrodynamic volume 10^3 times larger than the space occupied by unhydrated hyaluronic acid (having a partial specific volume of 0.66 ml/g; Varga, 1955), it can be calculated[2] that the decrease of 73 μg/g wet wt in the hyaluronic acid concentration of brain between 1 and 3 weeks of age would account for 82% (49 mg) of the 60 mg/g decrease in water content of brain during this same time period. It thus appears reasonable that changes in hyaluronic acid may be an important factor in the decrease in water content of brain during development, and indirectly in determining the size and nature of the extracellular space and the course of neuronal migration and differentiation.

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[2] Calculations have been based on values of 3.66 and 1.00 μmol of hyaluronic acid glucosamine per g of lipid-free dry weight, with 7.6 and 10.2% lipid-free dry weight, at 7 and 21 days, respectively. Glucosamine constitutes 43% by weight of hyaluronic acid.