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NET SULFATIDE SYNTHESIS, GALACTOSYLCERAMIDE SULFOTRANSFERASE AND ARYLSULFATASE A ACTIVITY IN THE DEVELOPING CEREBRUM AND CEREBELLUM OF NORMAL MICE AND MYELIN-DEFICIENT JIMPY MICE

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Summary

Net sulfatide synthesis, galactosylceramide sulfotransferase (EC 2.8.2.11) and arylsulfatase A (EC 3.1.6.1) activities were measured in two brain regions, cerebrum and cerebellum, of normal and jimpy mice during postnatal development.

In normally myelinating mice, two phases of increasing rates of net sulfatide synthesis were observed, the first coinciding with oligodendrocyte proliferation and the second with myelination. Net sulfatide synthesis was quantitatively higher in the cerebellum than in the cerebrum. In both brain regions, the developmental patterns of net sulfatide synthesis were related to the activity patterns of both galactosylceramide sulfotransferase and arlysulfatase A.

In jimpy mice, a neurological mutant showing hypomyelination in brain, the first phase of net sulfatide synthesis was preserved in both brain regions and galactosylceramide sulfotransferase and arylsulfatase A activities were normal up to 12 days. However, during the phase in which myelination occurred in controls, the net sulfatide synthesis in both brain regions of jimpy mice was zero or even negative. The sulfatide deficit was larger in the cerebellum than in the cerebrum. In both mutant brain parts, galactosylceramide sulfotransferase activity increased up to 12 days showing about 50% of the maximal activities observed in normal brain regions. Thereafter up to 15 days, enzyme activity decreased to about 25% of that of controls and remained low in both brain

Abbreviations: PAPS, 3′-phosphoadenosine 5′-phosphosulfate; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis(5-phenyloxazolyl-2)benzene.

regions. The developmental patterns and the activities of arylsulfatase A were, however, normal in the cerebrum and cerebellum of jimpy mice.

These results suggest that the enzyme activities and the developmental patterns of galactosylceramide sulfotransferase and arylsulfatase A as measured in vitro reflect to a high degree their functional activity in vivo. Furthermore, sulfatide degradation by arylsulfatase A seems to be important in regulating net sulfatide synthesis during normal and impaired myelination.

Introduction

In the myelinating mammalian brain, an important structural lipid of the myelin membranes, sulfatide [1], is synthesized from cerebroside and 3'-phosphoadenosine 5'-phosphosulfate by the enzyme galactosylceramide sulfotransferase, which is enriched in the microsomal fraction of brain [2,3]. After synthesis, sulfatide can be incorporated into myelin [4—6], where it shows a very low turnover rate with a half-life of more than 100 days [7]. On the other hand, sulfatide can be enzymatically degraded by cerebrosidesulfate sulfatase (EC 3.1.6.8) [8,9], usually measured as arylsulfatase A (EC 3.1.6.1.) activity, which is enriched in the lysosomes [10]. In a recent in vivo study on the sulfatide metabolism during normal mouse brain myelination, the existence of a sulfatide pool with a short half-life of only 1 day was demonstrated [11]. Thus, the parallelism of the developmental activity patterns of galactosylceramide sulfotransferase and arylsulfatase A, observed earlier in whole brains of normally myelinating mice [12], may reflect a cooperation of the metabolic function between the two enzymes.

In a neurological mutant, the jimpy mouse, brain myelination is impaired [13—17]. This is reflected by a reduced galactosylceramide sulfotransferase activity [18,19] and a strongly reduced rate of sulfatide biosynthesis [20] during development. This may lead to a reduced sulfolipid content as described for whole brain from 20-day-old jimpy mice [15]. The activity and the developmental pattern of the degrading enzyme aralysulfatase A was, however, normal in whole brain from jimpy mice up to 18 days [12,21].

Using normal mice and the dysmyelinating jimpy mouse mutant we tried to answer the question whether the galactosylceramide sulfotransferase and arylsulfatase A activity patterns determined in vitro express their functional activities in vivo. Both activities result in the net accumulation of sulfatide in the developing brain.

Since quantitative differences of sulfatide metabolism were noted previously in the cerebrum and cerebellum of normally myelinating mice [11], we studied these two brain regions of normal and jimpy mice separately.

Materials and Methods

Animals. Brown mice from the C57 BL/6J-A^{W-J} strain (Jackson Laboratories, Bar Harbor, ME, U.S.A.) were bred according to Wolf and Holden [22]. Normal males were used as controls to the hemizygous jimpy mice.

Chemicals. ATP, bovine cerebrosides and dipotassium 2-hydroxy-5-nitro-

phenyl sulfate were obtained from Sigma, bovine sulfatides from Applied Science and [^{35}S]PAPS from New England Nuclear. Azur A was purchased from Chroma, Triton X-100 from Mann, and Permablend® II (PPO 98%/POPOP 2%) from Packard. All other chemicals were purchased from Merck.

Sample preparation. After killing the animals of known age (days after birth) by decapitation, the cerebrum and cerebellum were isolated. The fairly pure cerebrum consisted of the two hemispheres. The cerebellum was freed of brain stem. The two brain areas were immediately weighed and either stored at −80°C or directly homogenized at 0°C in an all-glass homogenizer. The aqueous homogenates (5%, w/v) were sonicated for 30 s in ice/water at 50 W with an ultrasonicator (Sonifier B12, Branson sonic power, Danbury, CT). Appropriate dilutions for enzyme assays, sulfatide and protein determinations were prepared with cold distilled water.

Enzyme assays. Galactosylceramide sulfotransferase activity was determined as described previously [23]. The specific radioactivity of [^{35}S]PAPS was 0.66 Ci/mmol and a final assay concentration of 1.4 μM was used. Enzyme activity was expressed as pmole [^{35}S]sulfatide formed/h per mg protein. 1 pmol [^{35}S]-sulfatide corresponded to 2200 dpm.

Arylsulfatase A activity was measured in homogenates diluted approx. 500-fold according to a modified assay system of Worwood et al. [24]. Incubations were performed at 15°C and pH 5.3 using 0.01 M dipotassium 2-hydroxy-5-nitrophenyl sulfate as the substrate, and a buffer of low ionic strength (0.025 M sodium acetate containing 0.025 M NaCl).

Determination of sulfatide. Total homogenate lipids were extracted and washed according to Folch et al. [25] and dried under a stream of nitrogen. After mild alkaline hydrolysis with 0.2 N NaOH in methanol for 15 min at 37°C according to Flynn et al. [26], deacylated sulfogalactosyl diacylglycerols and saponified phospholipids were separated from sulfatides by means of partition in an aqueous-organic system followed by three washing steps [25]. The organic phase was dried under nitrogen and sulfatide was estimated by the colorimeteric assay of Kean [27]. In some experiments, the lipids in the chloroform phase were separated by thin-layer chromatography on silica gel-impregnated glass microfiber sheets (Type ITLC-SG of Gelman Instrument Company, Ann Arbor, MI, U.S.A.) using $CHCl_3/CH_3OH$ (87 : 8, v/v) for development. Sulfatide was quantitatively extracted from the gel by stepwise elution with $CHCl_3/CH_3OH$ (2 : 1 and 1 : 1, v/v) and methanol alone. After drying the combined extracts under nitrogen, sulfatide was determined according to Kean [27], using bovine sulfatides as reference.

Protein determination. The protein content was measured according to Lowry et al. [28] using crystallized bovine serum albumin as a standard.

Results

The protein content of the developing cerebrum and cerebellum of normal and jimpy mice. The total protein content of the cerebrum of normal and jimpy mice showed a similar increase from 3.8 mg (range ±5%) per cerebrum at birth to 30.0 mg (range ±8%) at 25 days. During the same developmental phase, the cerebellar protein increased from 1.5 mg (range ±6%) per cerebellum to

10.5 mg (range ±8%) in both normal and mutant mice. The developmental changes in protein content, expressed as mg protein per g fresh tissue, were similar in both brain regions of normal and mutant mice of the same age (Table I).

Galactosylceramide sulfotransferase activity patterns in the developing cerebrum and cerebellum of normal and jimpy mice. In both brain regions of normal mice, galactosylceramide sulfotransferase activity showed a biphase increase during the first 2 postnatal weeks (Fig. 1a). Throughout postnatal development, the specific activity was significantly higher in the cerebellum than in the cerebrum. Peak activities were observed in the cerebellum between 11 and 15 days, and in the cerebrum between 15 and 18 days. In the cerebrum and cerebellum of jimpy mice (Fig. 1b), galactosylceramide sulfotransferase activities showed an increase after birth with peak activities at 12 days in both brain parts. These activities were about 50% of the peak activities observed at 15 days in normal controls. At 15 days, enzyme activities dropped to about 25% of normal activities in both brain regions of jimpy mice.

Arylsufatase A activity patterns in the developing cerebrum and cerebellum of normal and jimpy mice. In both brain regions of normal mice (Fig. 2a), the developmental patterns of arylsulfatase A activity were comparable to those described for galactosylceramide sulfotransferase. From the 10th postnatal day onward, arylsulfatase A activity was higher in the cerebrum than the cerebellum. In both brain regions of jimpy mice (Fig. 2b), the activities as well as the developmental pattern were normal.

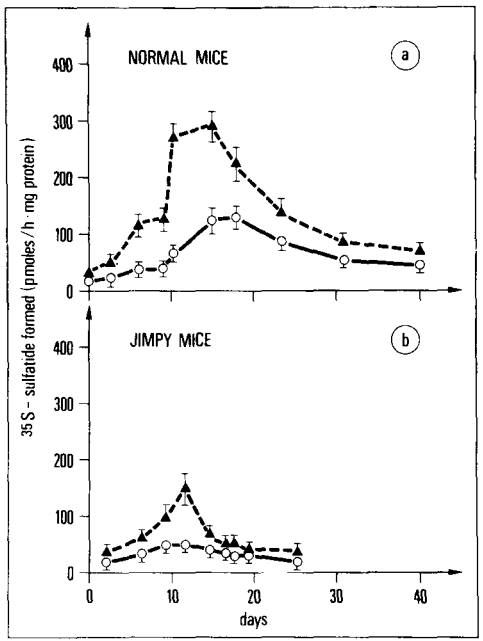
The sulfatide content of the developing cerebrum and cerebellum of normal and jimpy mice. In both brain regions of normal mice (Fig. 3a), two phases of sulfatide accumulation were observed, one during the first 10 days of life and a second after the 15th day. The sulfatide content was higher in the cerebellum than in the cerebrum. In the cerebrum and cerebellum of jimpy mice (Fig. 3b), a normal increase of the sulfatide content occurred during the first 10 postnatal days. But thereafter a decrease of the sulfatide content was observed in both brain regions of jimpy mice. This decrease in sulfatide content was greater in the cerebellum than in the cerebrum (Fig. 3a, b).

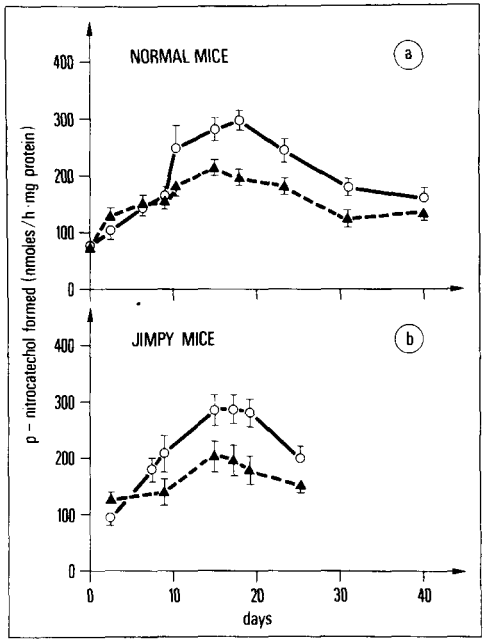
TABLE I

DEVELOPMENTAL CHANGES OF PROTEIN IN THE CEREBRUM AND CEREBELLUM OF NORMAL AND JIMPY MICE

All data are expressed as mg protein/g fresh tissue. Given are mean values and (1 S.D.) of 4—6 determinations.

Age in days	Cerebrum		Cerebellum	
	Normal	Jimpy	Normal	Jimpy
0	79 (5)	78 (3)	78 (1)	79 (3)
5	81 (4)	87 (5)	82 (5)	86 (7)
10	92 (2)	99 (5)	95 (5)	93 (7)
15	115 (2)	112 (3)	117 (3)	113 (4)
20	117 (7)	119 (4)	114 (5)	116 (4)
25	121 (6)	125 (7)	122 (4)	121 (8)
30	125 (4)	—	127 (6)	—
40	136 (2)	—	136 (3)	—





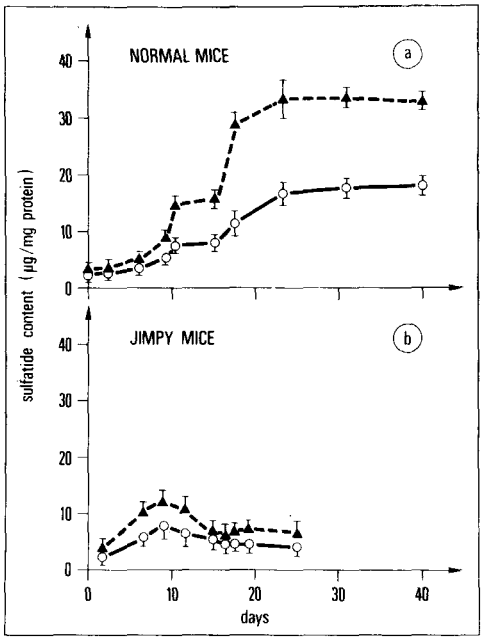


Fig. 1. Activity patterns of galactosylceramide sulfotransferase of the cerebrum and cerebellum of normal (a) and jimpy (b) mice during development. Enzyme activity was measured in aqueous homogenates (100—500 µg protein) using natural substrate (50 µM cerebrosides from bovine brain). 1,4 µM [^{35}S]PAPS (0.66 Ci/mmol), 0.4% Triton X-100 in 100 mM imidazole hydrochloride buffer of pH 6.5 containing 200 mM NaCl, 20 mM $CaCl_2$ and 5 mM ATP. The assay mixture (0.5 ml) was incubated at 30° C for 20 min [23]. All data plotted are mean values ±S.E. of 6—10 determinations. (▲- - - - -▲) cerebellum; (○———○) cerebrum.

Fig. 2. Activity patterns of arylsulfatase A of the cerebrum and cerebellum of normal (a) and jimpy (b) mice during development. Enzyme activity was measured in very dilute aqueous homogenates (less than 200 µg protein/ml) using *p*-nitrocatechol sulfate as the substrate. Incubations were performed at pH 5.3 and 15° C according to Worwood et al. [24]. Under these conditions, the enzyme reaction was linear with time and protein concentration for 1 h. All data plotted are mean values ±1 S.E. of 6—10 determinations. (▲- - - - -▲) cerebellum; (○———○) cerebrum.

Fig. 3. Development changes in the sulfatide content of the cerebrum and cerebellum of normal (a) and jimpy (b) mice. Sulfatide determinations were made in partially purified brain lipid mixtures after removal of sulfogalactosyl diacylglycerols and saponifiable phospholipids using the colorimetric assay for sulfolipids according to Kean [27]. All data plotted are mean values ±1 S.E. of 6—10 determinations. (▲- - - - -▲) cerebellum; (○———○) cerebrum.

Discussion

Sulfatide was measured by the method of Kean [27] in partially purified brain lipid mixtures after removal of sulfogalactosyl diacylglycerols and saponifiable phopsholipids. Sulfatide was also isolated by thin-layer chromatography before performing the Kean determination (for details see Materials and Methods). The two methods were used for sulfatide determination in both parts of brain from normal and jimpy mice aged 4 and 15 days, respectively, and comparable results were obtained. The values of net sulfatide synthesis obtained were comparable to those determined by Nonaka and Kishimoto [29] in different areas of rat brain using high-performance liquid chromatography for separation.

The net accumulation of sulfatide in the developing brain is the result of total sulfatide synthesis and degradation. A comparison of the developmental variation of the net sulfatide synthesis with the activities of the anabolic and catabolic enzymes in normal and mutant mice only becomes meaningful if the values taken for reference are similar and the enzymes to be compared are identical. Therefore, all parameters are referred to mg protein, since the protein content per g of cerebrum and cerebellum were the same in normal and jimpy mice at comparable developmental stages (see Table I).

The enzymes were measured using optimized assay conditions and interference by endogenous factors which change during development [12,23] could be excluded. Previous studies of the physico-chemical properties, such as pH optimum and heat inactivation of the two enzymes [12,30] indicated that the enzymes are identical in normal and jimpy mice brain.

In the normally myelinating cerebellum and cerebrum, the net sulfatide synthesis showed a biphasic developmental pattern. The same pattern has also described for two other important myelin constituents, the myelin basic proteins [31] and the cerebrosides [32]. The first phase of net sulfatide synthesis coincides with a burst of oligodendrocyte proliferation [33—35]. The second phase of high net sulfatide synthesis occurs between the 15th and 24th postnatal day (Figs. 3a, 4a, b) and coincides with the period of active myelination [36,37]. The developmental pattern of net sulfatide synthesis is the same in both brain parts, but the net synthesis is higher in the cerebellum both during oligodendrocyte proliferation and myelination (Fig. 4a, b). This suggests a higher rate of myelin synthesis in cerebellum than in cerebrum.

In normal mice, the developmental pattern of galactosylceramide sulfotransferase activity is also biphasic with a first shoulder before 9 days. The first part coincides with the first phase of net sulfatide synthesis in both brain regions (Fig. 4a, b). The developmental pattern of galactosylceramide sulfotransferase has been shown to reflect the changes of its functional activity in vivo [11,20,23,37,38]. Therefore, the observed decrease of net sulfatide synthesis to essentially zero between day 10 and 15 of postnatal life, inspite of a major increase of galactosylceramide sulfotransferase activity, has to be explained as being the result of increased catabolism, most likely by the action of arylsulfatase A which shows an increased activity during this time. It can be assumed that the degradation of sulfatide occurs exclusively in the lysosomes, in which arylsulfatase A is mainly located, since a genetic deficiency of this

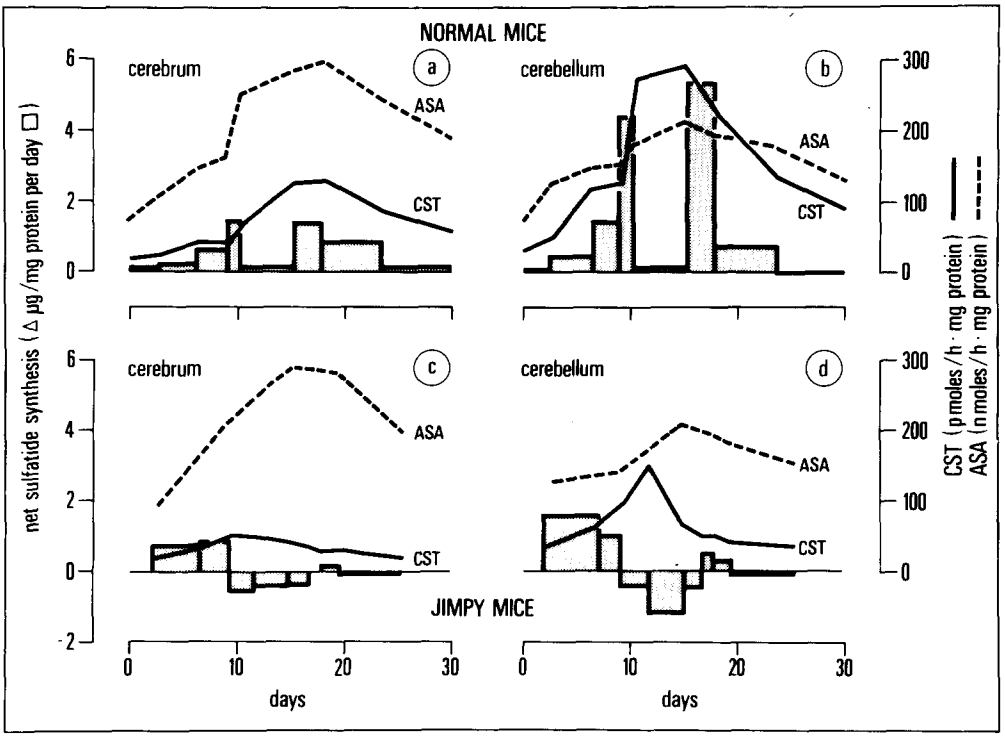


Fig. 4. Comparison of the developmental change of net sulfatide synthesis (bars) with the developmental activity patterns of galactosylceramide sulfotransferase (CST) and arylsulfatase A (ASA) in the normal cerebrum (a) and cerebellum (b) and in the jimpy cerebrum (c) and cerebellum (d). The rates of net sulfatide synthesis ($\Delta\mu$g sulfatide/mg protein per day) were derived from the data shown in Fig. 3, by dividing the difference of successive curve points by the corresponding time interval in days. To facilitate comparison with the anabolic and catabolic enzyme activities, the curves shown in Figs. 1 and 2 are repeated in this figure.

enzyme in man leads to abnormal accumulation of sulfatide in the lysosomes [9,39,40] already early in life.

In the developing cerebrum and cerebellum of jimpy mice, the net sulfatide synthesis and the activities of galactosylceramide sulfotransferase and of arylsulfatase A were close to normal during the first week of postnatal life (Figs. 3 and 4). Net sulfatide synthesis appears to be even higher in both the cerebrum and cerebellum of jimpy animals during the first week. After the 10th day, however, net sulfatide synthesis became zero or even negative in both brain regions (Fig. 4c, d). This may mean that catabolism prevails over synthesis. Galactosylceramide sulfotransferase activity (Fig. 1b) as well as total sulfatide synthesis measured in vivo by incorporation of $^{35}SO_4$ into sulfatide are low but not totally absent in jimpy mice brain during this period [20]. Thus, the observed negative balance of sulfatide metabolism in both brain regions of mutant mice (Figs. 3b, 4c, d) strongly suggests that arylsulfatase A is functionally active in jimpy mice and that the normal activity as measured in vitro corresponds to the functional activity in vivo.

Thus in the normal, as in the impared, myelination sulfatide degradation by

arylsulfatase A seems to be one important factor in regulation of net sulfatide synthesis.

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