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HORMONAL EFFECTS ON THE DEVELOPMENT OF RAT BRAIN GANGLIOSIDES—I. CORTISOL*

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Abstract—Ganglioside *N*-acetylneuraminic acid (NANA) content and distribution and that of incorporated radionuclide from 1-[^{14}C]glucosamine were investigated under conditions of normal development and after administration of 500 μg of cortisol at 1 day of age. Cortisol delayed significantly the rise in tissue concentration of NANA usually occurring between 2 and 20 days of age. Cerebellum was most severely affected and brainstem least. The incorporation of radionuclide was depressed during the developmental lag, then increased beyond controls from 6 to 10 days, and was again below that of controls in cerebrum and brainstem at 20 days. Individual study of seven chromatographically distinct resorcinol-positive compounds indicated that cortisol treatment favored accumulation and labeling of the more polar gangliosides initially, but resulted at 20 days of age in an abnormal ganglioside pattern with less polar compounds present disproportionately.

Recent studies have drawn attention to the gangliosides as possible surface receptors for extraneuronal substances. The history of this work indeed goes back at least to 1956, when Klenk [1] demonstrated the cleavage of *N*-acetylneuraminic acid (NANA) from gangliosides by a strain of influenza virus Bogoch [2] showed that the neurotoxic effect of influenza virus was inhibited *in vivo* by gangliosides. The fixation and activation of tetanus and botulinum toxins by gangliosides by a strain of influenza virus. Bogoch [2] quent year [3–5]. The idea that a single ganglioside, G_{M_1}§, was responsible for binding cholera toxin was suggested by King and van Heyningen [7] and has been studied further by others [8]. Since then, ganglioside interaction with hormones has been suggested [9]. Gangliosides have many of the properties necessary to the receptor role as formulated in the "mobile receptor theory" of Jacobs and Cuatrecasas [10].

Because of a concurrence of the period of rapid ganglioside increase in the brain with that of the proliferation of dendritic processes and synapses, the developmental pattern of brain gangliosides has been studied in several species, including human [6, 11, 12–15], rat [13, 15–19], chicken [18] and pig [19]. In the rat, total ganglioside NANA increases about 3-fold from birth to 20–25 days [17], the period of most rapid ganglioside increase being between 9 and 15 days. The concentration then stabilizes and actually decreases slightly by the age of 1 year [20].

Early studies of administration of cortisol to young rats [21, 22] found that hormone given on the first 4 days of life retarded the development of seizure responses to electric shock by 3 days, while cortisol injected on days 8–12 caused a 3-day acceleration in the emergence of the response. Schapiro *et al.* [23] reported on the effects of cortisol acetate (0.5 mg) given at 1 day of age to rats. The development of swimming ability and the sensorimotor cortical-evoked potential were delayed 2–3 days by cortisol. This work was confirmed by Salas and Schapiro [24] for evoked potentials in response to somesthetic, auditory and visual stimulation. This last paper reported that cortisol accelerated opening of the eyes by about 2 days, but noted that eye-opening involved basically a catabolic process. It also described electron microscopic evidence for a delay in maturation of brain dendritic spine processes in the cortisol-treated animals. This confirms earlier work of Schapiro [25], who found that 1 mg cortisol on day 1 of life decreased brain cholesterol, locomotor activity and body weight and delayed the appearance of dendritic spines in the cortex. These findings were in keeping with those of other investigators [26–28], who reported a greatly decreased rate of cell proliferation in the cerebrum and cerebellum of rats given 0.2 mg cortisol on the first 4 days of life. In our laboratory, cortisol has been found to delay the course of post-natal changes in levels of activity of ornithine decarboxylase [29].

The purpose of the present study was to establish a more extensive data base pertaining to the gangliosides during normal development, and also to describe the influence of neonatal cortisol administration. The developmental course was investigated by assay of endogenous gangliosides as well as through measurement of the incorporation of radioactive label from [^{14}C]glucosamine. Whole brains and three brain regions were studied.

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§ The nomenclature of Svennerholm [6] is used throughout.

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MATERIALS AND METHODS

Pregnant female albino rats were obtained from Zivic–Miller Corp., Allison Park, PA. Orders were arranged so that the animals arrived on or before day 18 of gestation. Litters, with lactating female, were housed in 35 × 20 × 12 cm metal cages or 47 × 25 × 20 cm plastic cages and given Purina Rat Chow and water *ad lib.* Beginning on the morning of day 20, animals were maintained under a condition of continuous light (in a white fluorescent-lighted room). This has been noted to have a synchronizing effect on parturition [30] and was utilized to promote the bearing of young during daytime hours. On day 21, nesting material was placed in each cage. The contents of the cage were not disturbed after the birth of pups, but animals were redistributed at 1 day of age to bring litter size within the range of eight to twelve pups. Retrospective analysis showed that the following factors did not influence results significantly: (1) season of the year, (2) sex of young, (3) litter size within stated limits, (4) size of cage (viz. two sizes mentioned above) and (5) interval between arrival and parturition.

Cortisol (Nutritional Biochemicals Corp., Cleveland, OH) was suspended in 10% ethanol at a concentration of 50 μg/μl. At 24 ± 1 hr of age, rats were injected with a single dose of 500 μg intraperitoneally, in a volume of 10 μl. Control animals received injections of vehicle. After injection, all animals were returned to the cage with their mother, and maintained until they reached the desired age.

Rats up to 28 days of age were injected intracranially with approximately 0.5 μCi of 1-[^{14}C]glucosamine (NEC 193X, New England Nuclear Corp., Boston, MA) in 10 μl of physiological saline, using a 2 or 3 mm × 27 gauge needle, depending on the size of the animal, and Hamilton 50-μl syringe (Hamilton Co., Whittier, CA). In animals more than 15 days old, light ether anesthesia was employed. With the head held immobile, the needle was inserted through the skull and normal to it over the mid portion of the left cerebral hemisphere, rostral to the transverse sinus and caudal to the main branch of the superior cerebral vein. The syringe was held in place for 5 sec to allow diffusion from the injection site and thus minimize loss of radioactivity. Care was taken to release pressure on the side of the head just before removing the needle, and the skin over the injection site was massaged briefly to aid in sealing the needle tract. Animals were then returned to their cages. All determinations were begun 24 hr after the injection of label, at which time incorporation into gangliosides is reported to be approaching its maximum [31]. Maccioni *et al.* [32] found that almost all the radioactivity from gangliosides labeled *in vivo* with [^{3}H]glucosamine was in the sialyl moieties.

Animals were stunned by a blow to the cervical spine, decapitated and the brains removed within 1 min. Immersion of the head in liquid nitrogen or killing by microwave irradiation was found not to improve the recovery of gangliosides. After decapitation, the brain was removed rapidly and, when appropriate, dissected into cerebral hemispheres, brainstem and cerebellum. The hemispheres included all cortical areas, basal ganglia and associated white matter, but not the thalamus. The cerebellum was separated by sharp sectioning of the peduncles flush with the underlying hindbrain. The term "brainstem" is applied to the remainder after removal of the above structures. Brain parts were pooled for analysis in order to provide 0.5 to 1.0 g tissue/tube. This required as many as fifteen parts (in the case of cerebellum at age 6 days). However, the number (N) reported here always represents the number of separate assays, rather than the number of individual parts. It was found that, at 3 days of age, cerebellar tissue of the rat was insufficient for ganglioside analysis. Therefore, this particular point in the data was omitted.

Gangliosides were prepared by a modification of the method of Suzuki [33]. Between 0.5 and 1.0 g of tissue was placed in 30 ml chloroform–methanol (2:1). Samples were homogenized on a Polytron homogenizer (PT10 or PT20, Kinematic, Lucerne, Switzerland),

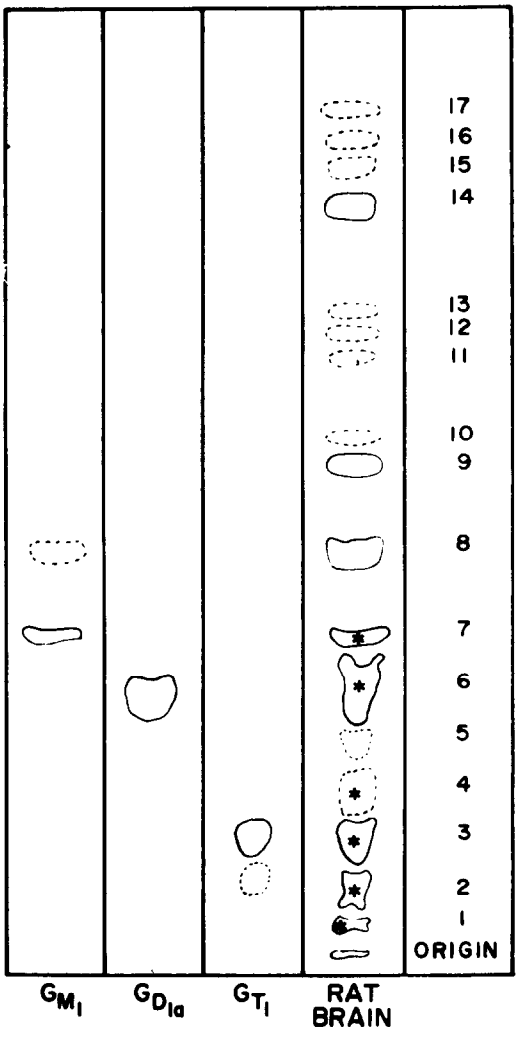

Fig. 1. Composite thin-layer chromatogram of rat brain gangliosides. Solid outlines indicate darker spots in iodine vapor than broken outlines, and asterisks are placed within those spots which are most strongly resorcinol-positive.

the homogenate was filtered through Whatman # 1 filter paper in a Buchner funnel under vacuum and the filtrate collected. The filter paper was removed rapidly from the funnel and the residue scraped from it. The residue was then rehomogenized in 15 ml chloroform–methanol (1:2) containing 5% water. Filtration was again carried out, this time until no further liquid issued from the funnel. The combined filtrates were reduced to dryness on a Flash evaporator (Buchler Instruments, Fort Lee, NJ) with the bath maintained at 33°. The lipid was re-dissolved in 10 ml chloroform–methanol (2:1) (re-distilled solvents) to which 2 ml of 0.88% KCl solution was added. Tubes were capped, mixed thoroughly on a Vortex (Genie K550-G: Scientific Industries Inc., Springfield, MA) mixer and then centrifuged at 1000 *g* for 20 min. The upper phase was removed and the lower phase washed first with 1.5 ml of pure solvent upper phase, and finally with 1.4 ml of pure solvent upper phase lacking KCl. The combined upper phases were transferred to 8-mm-dia. dialysis tubing and dialyzed against constantly flowing distilled, de-ionized water for 24 hr. The contents of the dialysis bag were then transferred to a tared tube, frozen to −40° and lyophilized. This lyophilized material constituted the ganglioside preparation. Internal controls consisting of previously prepared and assayed gangliosides were included in each run of the isolation and assay procedures.

When individual gangliosides were to be separated, samples were redissolved in chloroform–methanol–water (10:10:3), and 10 μl, containing 100–150 μg of lyophilized ganglioside preparation, was applied as a 3–4 mm dia. spot 1 cm above the edge of a 20-cm glass thin-layer chromatography (t.l.c.) plate coated with silica gel G (Brinkman Instruments Inc., Westbury, NY) containing no gypsum. Chromatograms were developed in standard glass tanks using chloroform–methanol–2.5 N NH_4OH (55:45:9) as solvent. Plates were run in duplicate and yielded chromatograms like that shown diagrammatically in Fig. 1: the loci of ganglioside standards (Supelco Inc., Bellafonte, PA) are also shown. There were seven resorcinol-positive spots. Labeled NANA, when chromatographed mixed with unlabeled preparation, remained at or near the origin. By comparison with standards and with known R_f values in several solvents, we identify spot # 2 with $G_{D_{3}}$, spot # 3 with G_{T_1}, spot # 4 with $G_{D_{1b}}$, spot # 6 with $G_{D_{1a}}$, and spot # 7 with G_{M_1}.

One duplicate plate was visualized by resorcinol spray and used as a template for confirming the location of spots on the other as visualized by iodine vapor. This procedure was followed because we found, in contrast to MacMillan and Wherret [34], that the resorcinol reaction could not be completely quantitatively after visualization by spraying with resorcinol reagent. After sublimation of the iodine, spots were scraped from the unstained plate and assayed for sialic acid content by a modification of the method of Jourdian *et al.* [35]. The procedure used in this laboratory was adapted for use on uneluted silica gel. Spots were scraped from t.l.c. plates into glass tubes and maintained at 0°. Next, 0.5 ml of 0.01 M periodic acid in 0.125 N H_2SO_4 was added and mixed gently by hand to avoid spreading the silica up the sides of the tube. The reaction was allowed to proceed for 35 min and terminated by addition of 1.25 ml of resorcinol reagent. This reagent was 0.6 g% resorcinol in 6.85 M HCl containing 0.25% 0.1 M $CuSO_4$. Each tube was mixed gently once again and kept at room temperature for at least 5 min to allow destruction of unreacted periodate, after which the color was developed at 110–115° for 15 min. Samples were cooled and 1.50 ml butyl acetate–n–butanol (85:15) was added. The tubes were mixed thoroughly and centrifuged at 600 *g* for 5–6 min. Optical density was measured against a reaction blank at 625 nm on a Zeiss model PNQII spectrophotometer (Carl Zeiss, Oberkochen, West Germany). The adaptation of this method for use with unelated silica gel did not decrease its sensitivity, and reproducible readings were obtained with samples containing as little as 10 nmoles NANA. The resorcinol-stained duplicate plate was used for the measurement of radioactivity. Samples were placed in glass vials and counted in 12 ml of a toluene fluor containing 4 g 2,5-diphenyloxazole (PPO) and 0.1 g 1,4-bis-2(5-phenyloxazolyl)-benzene (POPOP) (both Packard Instrument Co., Downey Grove, IL)/liter. Radioactivity was assayed using a Nuclear Chicago Mark II liquid scintillation spectrometer; efficiency was determined by means of channel check ration, and all data were presented as disintegrations per minute (dis./min).

Endogenous ganglioside levels were studied at 2, 6, 9, 10, 11, 13, 14, 15, 16 and 20 days of age and brain parts were studied at 6, 10, 11, 14, 16 and 20 days. Incorporation of radioactive label from [^{14}C]glucosamine as well as the distribution of NANA and label among chromatographically distinct entities was determined at 2, 6, 10, 15 and 20 days for the whole brain and in brain regions at 6, 10 and 20 days of age. Altogether, 122 whole brains and 202 dissected brains were studied under the cortisol condition, while 79 whole brains and 80 dissected brains served as controls. The controls were found to differ in no way from other large groups of normal animals studied.

RESULTS

Results of growth. Cortisol treatment on day 1 of life was associated with delayed growth in rats, as reflected by body weights and brain weights. The experimental group started out 0.50 g heavier than controls before treatment on day 1. Although the large N gives this statistical significance, it is thought to be a result of random variation. This group then actually lost weight in the subsequent 24-hr period (6.62 ± 0.05 g to 6.50 ± 0.07 g) while controls gained 0.62 g. Treated animals were 10–15 per cent lighter than controls between 3 and 10 days of age ($P < 0.005$) but appeared to catch up by 15 days and their weights remained normal through 20 days. Animals receiving cortisol had a mortality of 52 per cent, double that of controls. Brain weights were affected slightly by cortisol treatment: at 2 days, the weight of the whole brain was equal approximately to that of controls (103 per cent), but it dropped to 88 per cent ($P < 0.001$) at 6 days. The weight increased and approximated control levels by days 9–11, and surpassed the controls in the interval of 13–16 days (115 per cent, $P < 0.001$). At 20 days of age, whole brain weights of cortisol-treated and control rats were equivalent.

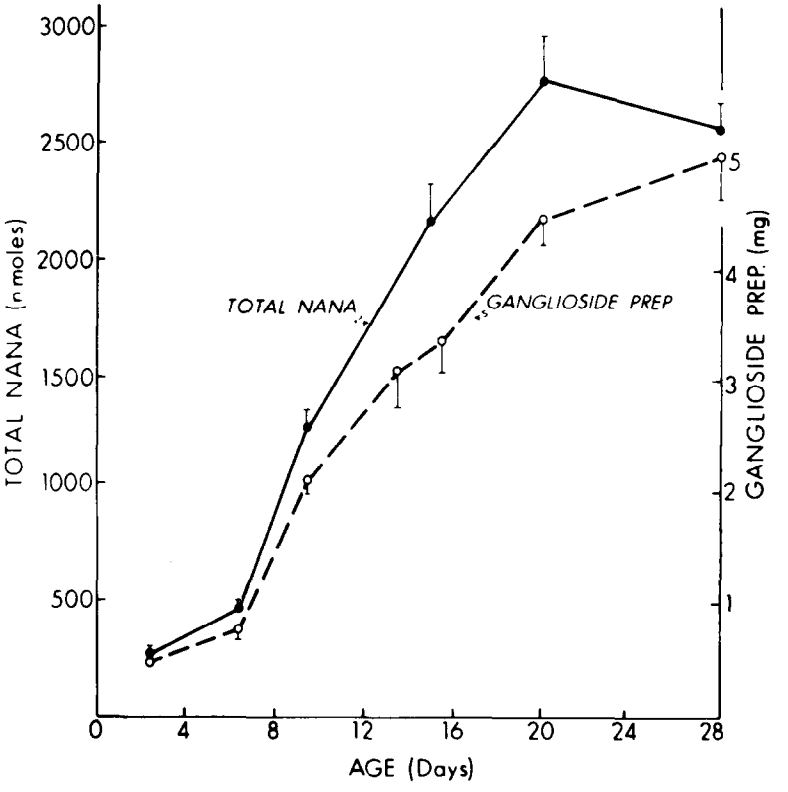

Fig. 2. Weight per brain of ganglioside preparation and value of its NANA content in whole brains of rats during development. Rats of the ages indicated were decapitated and the brains removed rapidly. Gangliosides were prepared as described in the text and NANA content was determined by the periodate–resorcinol method.

When the three brain regions were considered separately, it was seen that the decreased whole brain weight at 6 days was due largely to a delay in growth of the cerebellum (75 per cent of control, $P < 0.001$), which, however, grew to control level by 19 days. The increased weight during the first half of week 3 of life and the return to normal by its conclusion were reflected in all brain parts.

Endogeneous total ganglioside. The endogenous level of gangliosides in brain was estimated by sialic acid content. The weight of the ganglioside preparation was also used as an indicator, and paralleled the NANA level closely. Normal data are shown in Fig. 2: each point represents ten to fifteen separate assays. The rate of ganglioside increase was greatest between 7 and 10 days of age. At 28 days, the ganglioside content had not changed significantly during the previous week. Growth curves for ganglioside preparation and NANA are compared for experimental and control animals on the basis of tissue wet weight in Figs. 3 and 4. At 2 and 6 days, values for experimental and control animals were equivalent. This was so despite a difference in absolute ganglioside content, because of the significant retardation of brain weight increase in the treated group. After 6 days, ganglioside preparation per gram of brain began to fall below controls and remained so throughout the duration of the study. The differences were significant at 9, 14–16 and 20 days of age. Ganglioside NANA was also apparently consistently less than in controls, but was statistically significant only at 16 days (83 per cent, $P < 0.05$) and had recovered almost completely by 20 days (90 per cent, NS). This systematic variation in NANA/mg of preparation suggests an alteration in the distribution of NANA among the various gangliosides.

Data on the ganglioside NANA content of brain regions are given in Fig. 5. Each value represents five to eleven separate assays performed on at least two different occasions. In normally developing cerebral hemispheres, NANA content increased 7-fold, an increase of almost 3-fold on the basis of tissue weight. The brainstem was richer significantly in ganglioside NANA than the cerebral hemispheres at 3 days of age ($P < 0.001$), but its rate of increase slowed after 6 days of age and was soon surpassed. On a tissue weight basis, the brainstem had reached its plateau by 10 days postpartum. The cerebellum, which was not developed sufficiently to afford measurement at 3 days, contained at 6 days a concentration of NANA not different significantly from that of the other brain parts. This level then dropped, however, and at 10

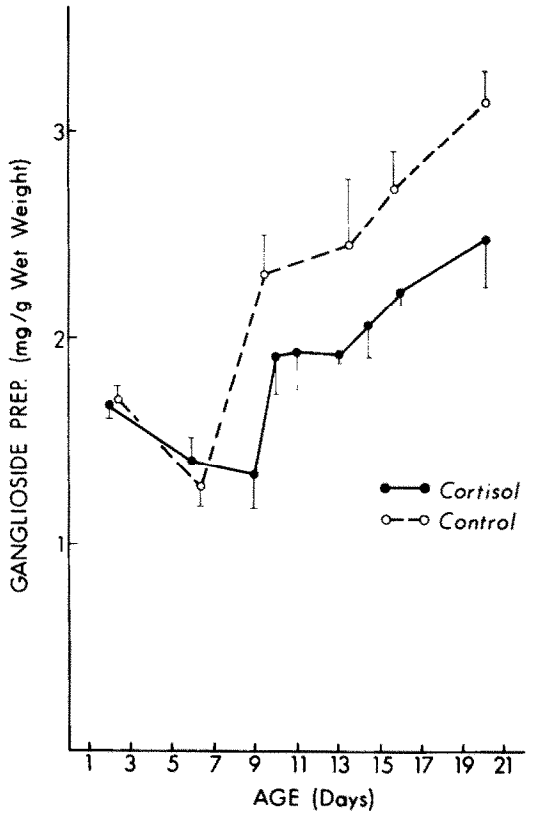

Fig. 3. Weight of ganglioside preparations per gram wet weight of brain tissue from rats given 500 μg cortisol i.p. at 1 day of age and from controls. Rats of the ages indicated were decapitated and the brains removed rapidly. Gangliosides were prepared as described in the text.

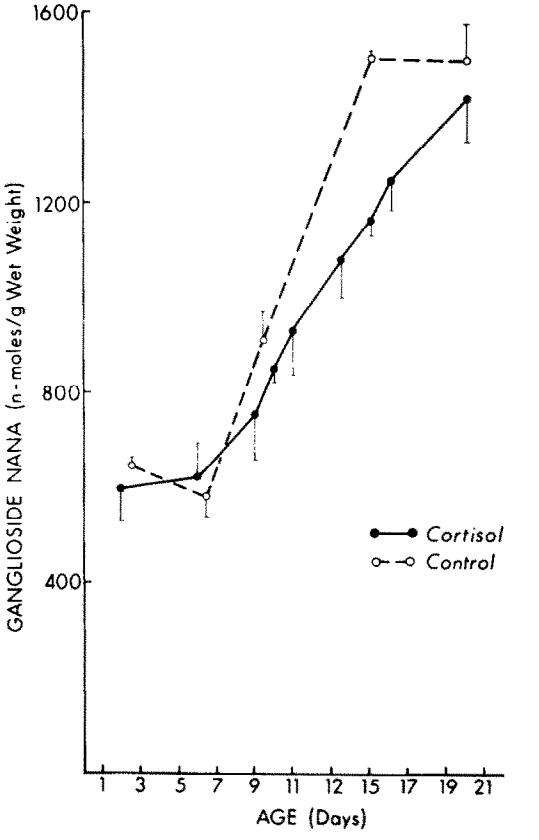

Fig. 4. NANA content of ganglioside preparation per gram wet weight of brain tissue from rats given 500 μg cortisol i.p. at 1 day of age and from controls. Rats of the ages indicated were decapitated and the brains removed rapidly. Gangliosides were prepared as described in the text and NANA content was determined by the periodate–resorcinol method.

days was significantly below the other regions ($P < 0.05$). This drop was replicated several times and was the only portion of the three curves having a negative slope. Figure 5 shows that this represents negligible net synthesis of NANA in the cerebellum over a 4-day period. At 10 days, cerebellar NANA began a period of rapid increase, bringing it within statistical range of the other parts by 20 days.

The effects of cortisol on ganglioside NANA content and concentration differed among the three brain regions studied. These data, as percentages of control values, are presented in Table 1. In cerebral hemispheres, the endogenous ganglioside level was equal to or slightly below control, particularly in the last interval between 15 and 20 days of age. This reflected the findings for whole brain. In the brainstem, there was a depression of approximately 35 per cent in NANA content at 6 days, on both an absolute and a tissue concentration basis, but no such difference from controls in the ganglioside preparation obtained, suggesting an alteration in ganglioside pattern (v.i.). After 6 days of age, there were no further statistically significant differences in ganglioside content between brainstem of cortisol-treated rats and that of controls. In the cerebellum, there was no diminution in ganglioside content at 6 days of age, but at 19 days, when the cerebellar weight had recovered to control levels, ganglioside content was about half that of controls, on both an absolute and concentration basis. After this, gangliosides accumulated rapidly, reaching control levels at 15 and 20 days of age (see Table 1).

Total ganglioside radionuclide incorporation. Data on the incorporation of radionuclide from labeled glucosamine into control specimens are represented in Fig. 6. Uptake of label by whole brains rose rapidly from 3.3 ± 0.3 per cent of the injected dose at 2–3 days to 5.6 ± 0.5 per cent at 10 days. It then fell even more steeply to 1.8 ± 0.1 per cent of the injected dose at 20 days. The brainstem peaked earlier, while cerebral hemispheres peaked at 10 days, and the incorporation of radionuclide into cerebellar gangliosides increased over the entire period studied. Although NANA distribution paralleled closely the weights of brain parts, the relationship between distribution of radioactivity and weight is at times not even ordinal. There appears to be a pattern of the cerebrum having incorporated more than its proportion by weight through 10 days of age and the cerebellum accounting for less than its proportion throughout the period studied.

The incorporation of radionuclide by brains of

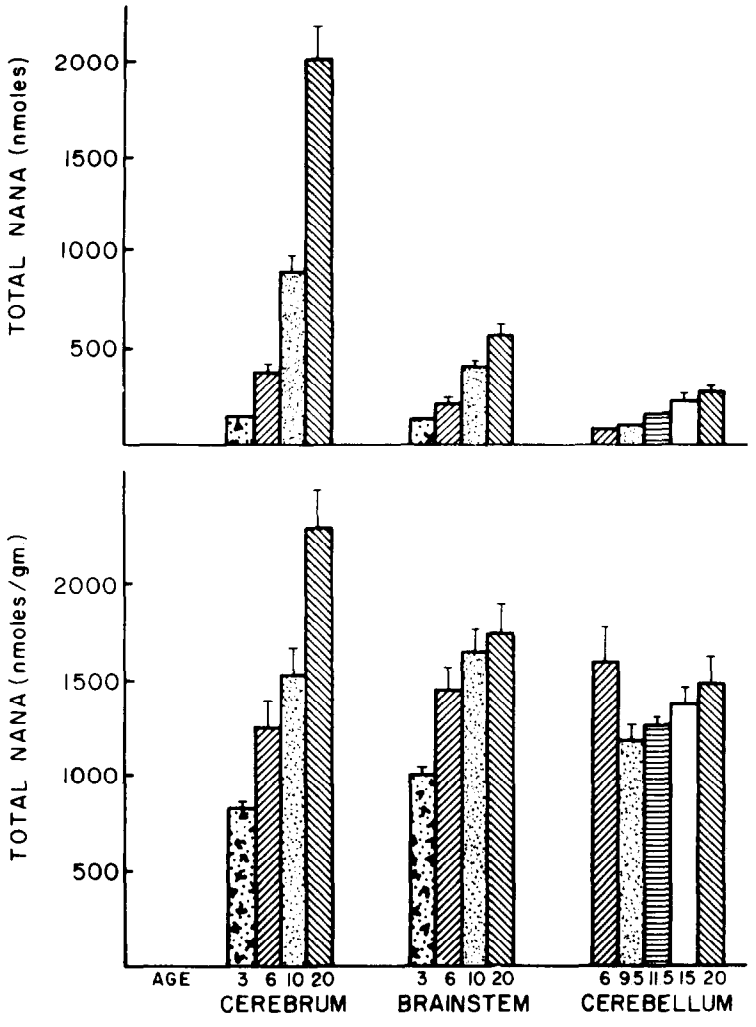

Fig. 5. Ganglioside NANA per brain part and per gram wet weight of tissue in three brain regions of developing rats. Rats of the ages indicated were decapitated and the brains removed rapidly and dissected. Gangliosides were prepared as described in the text and assayed by the periodate–resorcinol method. Values are means ±S.E.M.

Table 1. Total ganglioside NANA of brain regions in cortisol-treated vs control rats*

	Per cent of control—per part (per gram)				
	Age (days)				
	6	10	11	14–16	20
Cerebral hemispheres	93 (96)	82 (83)	84 (81)	84 (70)†	96 (95)
Brainstem	63‡ (65)§	95 (102)	105 (107)	121 (108)	95 (90)
Cerebellum	84 (111)	55‖ (45)‖	65‖ (50)‖	102 (88)	115 (114)

* Experimental animals received 500 μg cortisol i.p. at 1 day of age. Each value represents six to twelve assays of the dialyzed, lyophilized Folch upper phase fractions of a total lipid extract.

† $P < 0.05$.

‡ $P < 0.005$.

§ $P < 0.01$.

‖ $P < 0.001$.

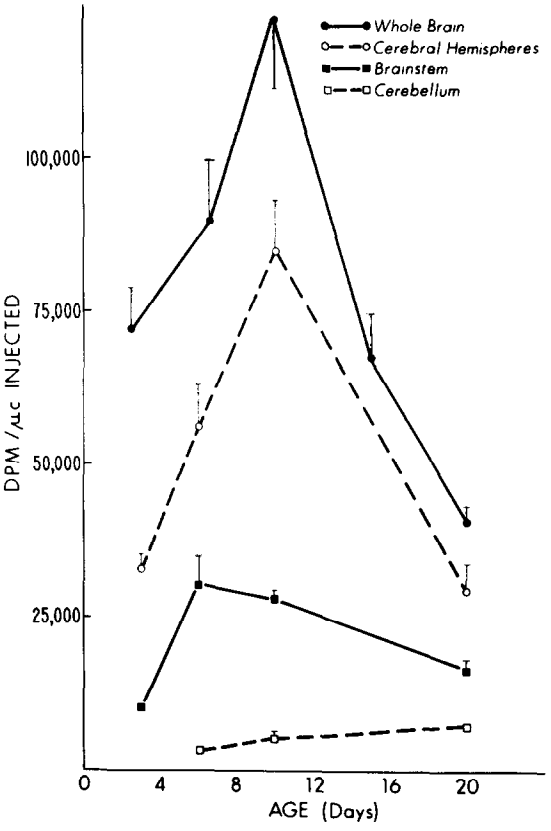

Fig. 6. Incorporation of radioactive label from 1-[^{14}C]-glucosamine over a 24-hr period following intracranial injection, in whole brains and three brain regions of developing rats. Rats of the ages indicated were decapitated and the brains removed rapidly. Gangliosides were prepared as described in the text and radioactivity was determined by liquid scintillation spectrometry.

cortisol-treated rats is represented in Table 2. In whole brain, the incorporation of radioactivity peaked at 6 days, rather than at 10 days. Also, after being depressed relative to controls at 2 days of age, the labeling increased strikingly by 6 days, achieving a value of 293 per cent of control ($P < 0.001$). While still elevated significantly at 10 days of age, the value had dropped considerably toward the control curve, and at 20 days, the whole brain incorporation of label was indistinguishable from controls. Investigation of brain regions revealed that increased incorporation of label at 6 and 10 days of age was equally characteristic of cerebrum, brainstem and cerebellum. At 20 days, however, the normal values for whole brain reflected slightly increased labeling in the cerebral hemispheres and decreased uptake of label in the brainstem and cerebellum.

Developmental course of seven individual resorcinol-positive materials: control animals. The percentage distribution of NANA among the seven resorcinol-positive spots is summarized graphically for normal whole brains in Fig. 7, and the NANA content of each spot during development is given in Table 3. The distribution of incorporated radioactivity is shown in Fig. 8 for whole brains and in Fig. 9 for brain parts.

The NANA content of the slowest-moving spot (# 1) increased approximately linearly from 6 to 20 days of age, reaching a wet-weight concentration of 105 ± 5 nmoles/g. Its percentage of the total NANA was stable at between 3 and 5 per cent. The concentration was relatively greater in brainstem at 3 and 6 days (not statistically significant) and in cerebrum at 10 and 20 days (84 ± 11 nmoles/g of cerebrum as compared to 48 ± 9 nmoles/g of brainstem, $P < 0.05$ at 20 days). In the cerebellum, spot # 1 decreased both absolutely and on the basis of weight between 6 and 10 days; the large variation prevented statistical

Table 2. Incorporation of label from intracerebrally injected [^{14}C]glucosamine into brain ganglioside of cortisol-treated rats during development*

	Age (days)				
	2	6	10	15	20
Whole brain					
(dis./min/brain/μCi injected)	46,211 ± 4,362	257,572 ± 23,663	162,650 ± 16,143	85,026 ± 8,947	39,301 ± 3,115
(% Control)	66†	293‡	133§	129‖	97‖
Cerebral hemispheres					
(dis./min/part/μCi injected)	×	135,102 ± 12,562	116,725 ± 9,175	×	30,942 ± 3,343
(% Control)		240‡	133§		100‖
Brainstem					
(dis./min/part/μCi injected)	×	108,100 ± 7,867	44,028 ± 3,328	×	6,670 ± 822
(% Control)		352‡	161†		50¶
Cerebellum					
(dis/min/part/μCi injected)	×	8,472 ± 940	7,282 ± 1,150	×	2,223 ± 277
(% Control)		268‡	142‖		41¶

* Experimental animals received 500 μg cortisol i.p. at 1 day of age. Each value represents 12–26 separate assays for whole brain and 8–12 assays for brain regions.

† $P < 0.01$.

‡ $P < 0.001$.

§ $P < 0.05$.

‖ Not significant.

¶ $P < 0.005$.

Table 3. Pattern of ganglioside NANA distribution in the rat brain during development*

Spot	NANA (nmoles) Age (days)				
	2–3	6–7	10	15	20
Whole brain					
Origin	15 ± 1	7 ± 1	14 ± 3	68 ± 11	61 ± 8
#1	11 ± 1	15 ± 3	46 ± 3	95 ± 11	149 ± 20
#2 (G_Q)	15 ± 1	30 ± 4	76 ± 8	111 ± 8	252 ± 26
#3 (G_{T_1})	55 ± 5	120 ± 12	350 ± 38	616 ± 30	878 ± 49
#4 ($G_{D_{1B}}$)	31 ± 3	49 ± 4	111 ± 23	188 ± 30	208 ± 27
#5	16 ± 3	22 ± 3	95 ± 12	132 ± 8	272 ± 32
#6 ($G_{D_{1A}}$)	46 ± 3	174 ± 12	446 ± 14	680 ± 65	722 ± 76
#7 (G_{M_1})	74 ± 4	66 ± 4	136 ± 9	204 ± 42	224 ± 35
Cerebral hemispheres					
Origin	4 ± 1	3 ± 1	12 ± 1	×	23 ± 3
#1	3 ± 1	6 ± 2	24 ± 3	×	74 ± 10
#2 (G_Q)	7 ± 1	14 ± 3	20 ± 4	×	108 ± 15
#3 (G_{T_1})	23 ± 2	51 ± 5	150 ± 17	×	454 ± 56
#4 ($G_{D_{1B}}$)	13 ± 2	20 ± 6	48 ± 7	×	107 ± 10
#5	7 ± 1	14 ± 1	45 ± 1	×	128 ± 15
#6 ($G_{D_{1A}}$)	19 ± 0	83 ± 10	219 ± 20	×	408 ± 41
#7 (G_{M_1})	28 ± 2	30 ± 7	77 ± 10	×	128 ± 11
Brainstem					
Origin	2 ± 0	3 ± 1	5 ± 1	×	7 ± 1
#1	4 ± 1	6 ± 2	8 ± 3	×	16 ± 3
#2 (G_Q)	5 ± 0	5 ± 2	18 ± 3	×	37 ± 9
#3 (G_{T_1})	17 ± 2	33 ± 3	77 ± 10	×	173 ± 24
#4 ($G_{D_{1b}}$)	10 ± 1	12 ± 2	26 ± 3	×	48 ± 7
#5	7 ± 1	5 ± 1	18 ± 4	×	42 ± 9
#6 ($G_{D_{1a}}$)	14 ± 0	42 ± 7	72 ± 8	×	111 ± 12
#7 (G_{M_1})	24 ± 2	11 ± 2	20 ± 3	×	27 ± 6
Cerebellum					
Origin	×	5 ± 2	4 ± 1	×	2 ± 1
#1	×	5 ± 2	2 ± 1	×	12 ± 2
#2 (G_Q)	×	4 ± 1	6 ± 1	×	27 ± 5
#3 (G_{T_1})	×	9 ± 1	20 ± 2	×	62 ± 9
#4 ($G_{D_{1b}}$)	×	4 ± 1	10 ± 1	×	14 ± 2
#5	×	4 ± 1	8 ± 1	×	16 ± 3
#6 ($G_{D_{1a}}$)	×	10 ± 1	20 ± 1	×	51 ± 6
#7 (G_{M_1})	×	8 ± 2	12 ± 1	×	12 ± 3

* Untreated rats of the ages indicated were decapitated and the brains removed rapidly. Gangliosides were prepared and thin-layer chromatography was performed in chloroform–methanol–2.5 N NH_4OH (55:45:9). Each resorcinol-positive spot was scraped and its sialic acid content determined by the periodate–resorcinol method. Each value represents ten to fourteen separate assays for whole brain and six to eight assays for brain regions.

significance. Incorporation of radioactivity into spot #1 followed a typical pattern with a peak at 10 days reflecting cerebral hemispheres, while brainstem incorporation showed less peaking and was maximal at 6 days, and cerebellar incorporation into spot #1 increased throughout the study. The relative proportion of dis./min in spot #1 was 1–3 per cent.

The sialic acid of spot #2, which corresponds to G_Q, was equal to 15 ± 1 nmoles/brain at 2–3 days, increasing slowly to 15 days, and then more rapidly to 252 ± 26 nmoles at 20 days. This represented a rapid change in wet weight concentration ($P < 0.001$). The proportion of total NANA attributable to spot #2 was stable up to 15 days, then jumped to 9.1 ± 1.3 per cent at 20 days ($P < 0.05$, Fig. 7). In brain regions, the rise in NANA concentration was significant in brainstem between 6 and 10 days (32 ± 13 to 72 ± 13 nmoles/g, $P < 0.05$), and in cerebrum and cerebellum between 10 and 20 days (34 ± 122 ± 18 nmoles/g, $P < 0.001$ and 76 ± 13 to 149 ± 38 nmoles/g, $P < 0.05$ respectively). The same relations held true when the proportion of total sialic acid was considered. The NANA per gram of tissue for spot #2 was highest in cerebellum at every point studied. Incorporation of radioactive label into spot #2 showed the usual peak at 10 days. There was an actual decrease in incorporation between 2–3 and 6–7 days, reflected by a significant drop in percentage ($P < 0.001$), and this was

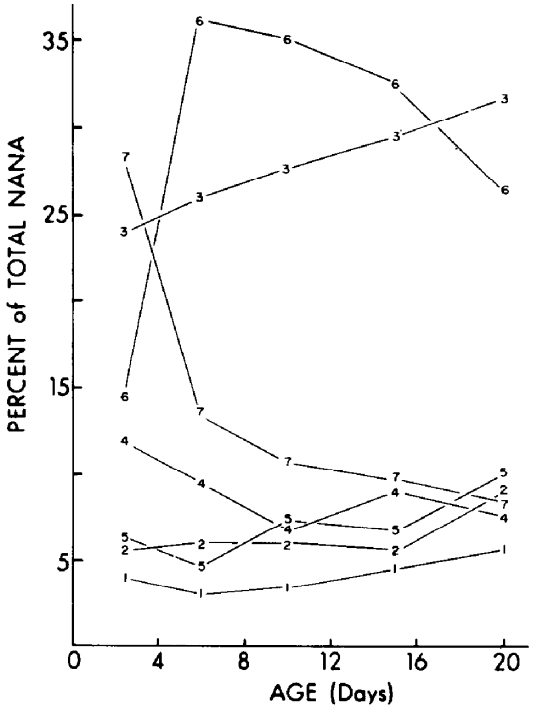

Fig. 7. Percentage distribution of NANA among individual chromatographic spots in whole brain of developing rats.

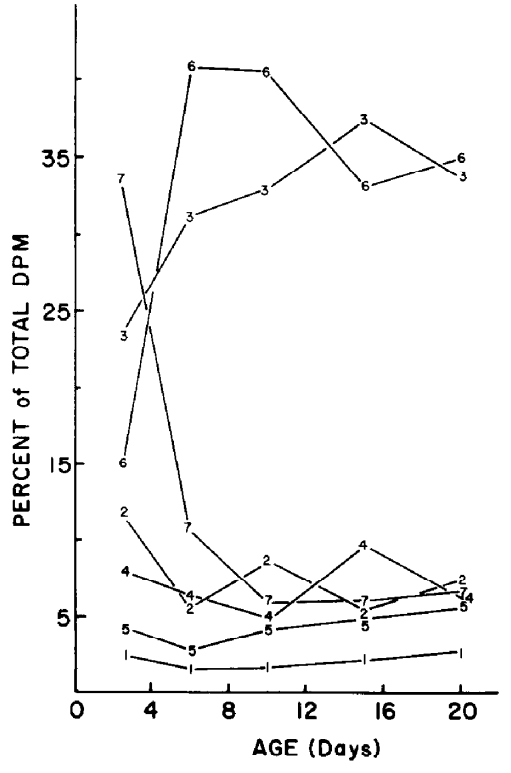

Fig. 8. Percentage distribution among individual chromatographic spots of radioactivity incorporated 24 hr after intracranial injection of 10^6 dis./min [^{14}C]glucosamine in whole brain of developing rats.

mostly accounted for by the cerebral hemispheres (12 ± 1.0 per cent at 3 days to 7.0 ± 0.7 per cent at 6 days, $P < 0.001$). Brainstem fluctuated less, varying irregularly between 5 and 10 per cent of the total dis./min. Incorporation in the cerebellum rose throughout the study and was highest among the brain parts, as a proportion of total dis./min.

Spot #3 represents trisialoganglioside, G_{T_1}. The NANA content doubled between 2–3 and 6–7 days and increased 7-fold between 6–7 and 20 days, reaching 878 ± 49 nmoles/brain. When compared on a molar basis to the other three major brain gangliosides, at 20 days of age, spot #3 accounted for 20 per cent of the four major gangliosides in brainstem, 28 per cent in cerebral hemispheres, and 32 per cent in cerebellum. Incorporation of radioactivity into spot #3 was typical, showing a peak at 10 days: the greatest proportional increase was between 2–3 days and 6–7 days ($P < 0.001$), with little further gain.

Spot #4 had a mobility identical with $G_{D_{1b}}$ of Svennerholm. The NANA of spot #4 increased from 2–3 to 20 days, the greatest change being between 6–7 and 15 days ($P < 0.05$). This increase, however, was slower than that of total brain gangliosides, reflected in a gradual drop in percentage of total NANA ($P < 0.005$, Fig. 7). The radiolabeling of spot #4 showed a broader developmental peak than that of other gangliosides. Its percentage of the total incorporation remained near 7 per cent throughout the first 20 days of life (Fig. 8). The separate brain parts were similar to whole brain, except cerebellum, where the NANA concentration on a weight basis peaked at 10 days and was significantly smaller both before and afterward ($P < 0.05$ and < 0.025 respectively). This unusual peak was also derived in terms of percentage of total ganglioside NANA, which reached 12 ± 1 per cent at 10 days and then dropped back to 7 ± 2 per cent at 20 days, which was indistinguishable from the rest of the brain. When compared with the other major brain gangliosides, $G_{D_{1b}}$ (spot #4), accounted for 10–15 per cent uniformly among the parts and throughout the first 3 weeks of development. Radiolabeling of cerebellar spot #4 represented 19 ± 0.8 per cent of the total cerebellar radioactivity at 6 days, significantly higher than that of the other brain parts ($P > 0.001$).

Spot #5 was a weakly resorcinol-positive spot frequently not visualized by iodine vapor. Its NANA content increased most rapidly in the last interval studied, reaching 273 ± 32 nmoles at 20 days. In its incorporation of radioactive label, spot #5 showed a sharp peak at 10 days, while the percentage of the total activity was uniform at about 5 per cent (Fig. 8).

Spot #6 was a large spot, which stained darkly with resorcinol reagent and corresponding to the major brain disialoganglioside, $G_{D_{1a}}$. Its NANA content rose almost 16-fold between 2 and 20 days of age. The most rapid rate of development occurred between 6–7 and 10 days. On the basis of brain weight, development of this ganglioside also was early, its concentration and percentage of total NANA more than doubling from 2–3 to 6–7 days ($P < 0.001$, Fig. 7). After 10 days, the rate of increase slowed and the concentration eventually leveled off, while the percentage of total NANA decreased

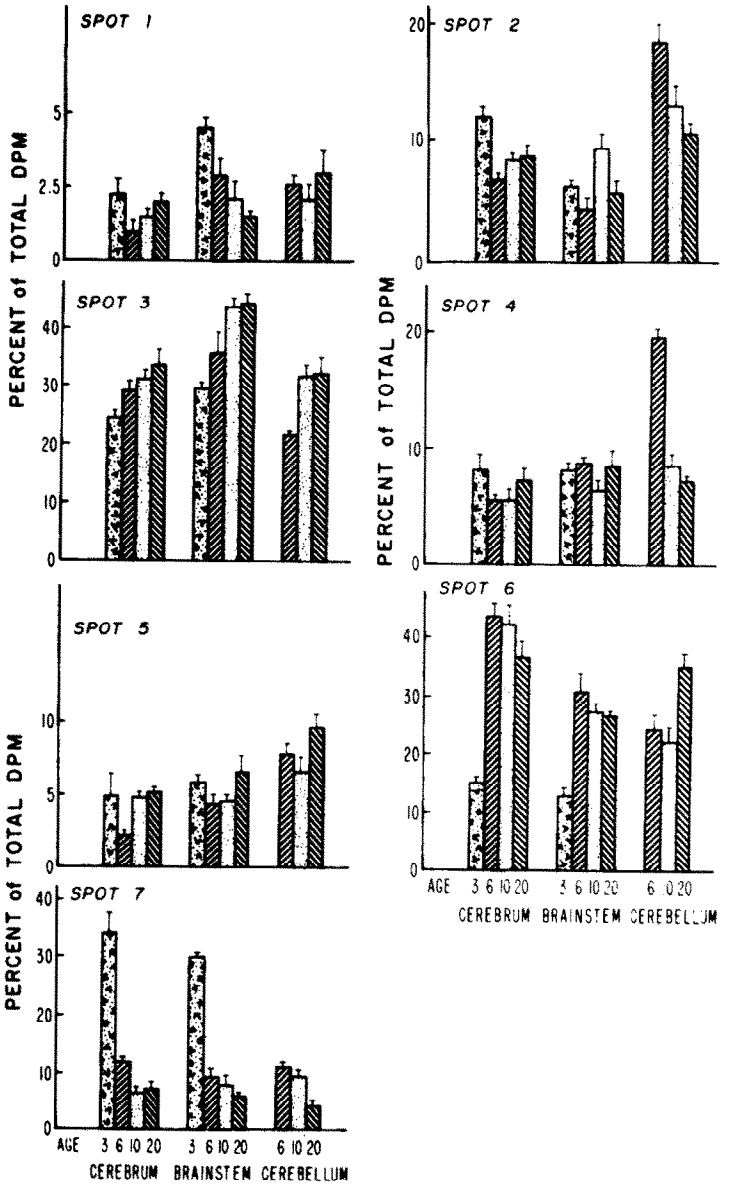

Fig. 9. Percentage distributions of radioactivity from injected [^{14}C]glucosamine among seven resorcinol-positive bands obtained by thin-layer chromatography of a ganglioside preparation from three regions of the rat brain. Twenty-four hr after intracranial injection of 10^6 dis./min 1-[^{14}C]-D-glucosamine, rats of the ages indicated were decapitated and the brains removed and dissected. Gangliosides were obtained as described in the text and separated by thin-layer chromatography in chloroform–methanol–2.5 N NH_4OH (55:45:9). Radioactivity was determined by liquid scintillation spectrometry. Values are means ± S.E.M.

slowly. There were no significant differences in spot #6 NANA concentration among brain parts at any of the ages measured. When the four major gangliosides are considered separately, $G_{D_{1a}}$ (spot #6) more than doubled its molar percentage in cerebral hemispheres and brainstem between 3 and 6 days of age (19 to 42 and 16 to 43 per cent respectively) while in cerebellum this increase occurred later. The uptake of label from glucosamine by spot #6 ganglioside was typical, and was consistent in magnitude with the early and rapid development of this material. The percentage of the total radioactivity found in spot #6 evinced a developmental pattern very similar to that of endogenous NANA.

Spot #7 represented the major monosialoganglioside G_{M_1}. Its NANA was stable between 2–3 and 6–7 days in whole brain, then tripled in amount by 15 days and was stable thereafter. In terms of tissue

Table 4. Ganglioside NANA in brains of cortisol-treated vs control rats*

	NANA content (per cent control) Age (days)				
	2–3	6–7	10	15	20
Whole brain					
# 1	75†	70	94	26‡	64†
# 2 (G_Q)	78	100	98	75	70†
# 3 (G_{T_1})	137†	98	82	110	82
# 4 ($G_{D_{1b}}$)	45†	67†	76	49†	48§
# 5	75	72	86	73	45
# 6 ($G_{D_{1a}}$)	123‖	95	90	138‖	141†
# 7 (G_{M_1})	75	88	97	150†	154†
Cerebral hemispheres					
# 1	×	45	50§	×	45‖
# 2 (G_Q)	×	86	155	×	76
# 3 (G_{T_1})	×	100	86	×	72
# 4 ($G_{D_{1b}}$)	×	60	73	×	44‡
# 5	×	80	96	×	82
# 6 ($G_{D_{1a}}$)	×	93	88	×	142
# 7 (G_{M_1})	×	70	81	×	131
Brainstem					
# 1	×	30	130	×	106
# 2 (G_Q)	×	72	98	×	89
# 3 (G_{T_1})	×	90	86	×	68
# 4 ($G_{D_{1b}}$)	×	52‖	80	×	48†
# 5	×	72	62	×	52
# 6 ($G_{D_{1a}}$)	×	58†	101	×	116
# 7 (G_{M_1})	×	156§	184‖	×	159
Cerebellum					
# 1	×	36	80	×	92
# 2 (G_Q)	×	82	54§	×	56†
# 3 (G_{T_1})	×	105	52§	×	98
# 4 ($G_{D_{1b}}$)	×	90	56†	×	57†
# 5	×	69	30‡	×	63†
# 6 ($G_{D_{1a}}$)	×	112	52‡	×	192†
# 7 (G_{M_1})	×	103	34‡	×	217‖

* Experimental animals received 500 μg cortisol i.p. at 1 day of age. Each value represents six to twelve separate assays.
† $P < 0.05$.
‡ $P < 0.001$.
§ $P < 0.01$.
‖ $P < 0.005$.

weight, this signifies a sharp fall from 248 ± 14 nmoles/g at 2–3 days to 122 ± 8 nmoles/g at 6–7 days ($P < 0.01$, Fig. 9). The fall in spot # 7 NANA occurred later in cerebellum than in the other parts. Consideration of the four major brain gangliosides shows clearly the fall in G_{M_1} (spot # 7) from very high molar percentages (53 and 56 per cent in cerebrum and brainstem at 3 days old, respectively, and 44 per cent in cerebellum at 6 days of age) to rather uniform percentages at the end of week 3 of life (16–24 per cent). The incorporation of label was highest at 2–3 days, and declined consistently throughout the period studied. Cerebellar incorporation actually increased somewhat between 6 and 10 days.

Developmental course of seven individual resorcinol-positive treated animals. The distribution of NANA among the seven resorcinol-positive spots separated chromatographically from brain and brain parts of cortisol-treated rats is compared with that for control animals in Table 4.

At 2 days of age there was a significant increase relative to controls in both the amount and proportion of NANA contained in spots # 3 and 6 and a decrease in spot # 4. At 6 days, the NANA distribution was similar to that of controls, except that the decrease in spot # 4 was maintained. Among brain parts, cerebral hemispheres showed an increased proportion of NANA found in spot # 7 and an overall decrease in spots # 4 and 6. Cerebellum, at 6 days, showed the pattern of increased sialic acid in spots # 3 and 6 which had been demonstrated in other parts at 2 days of age. At 10 days of age the slight diminution measured in total ganglioside NANA in whole brain was distributed nearly evenly over the individual gangliosides and, separately, none differed significantly from control. The brain regions each gave a distinct picture, which would have been obscured had only whole brain been studied. In the cerebrum, there was slightly less ganglioside than in controls, particularly in spot # 1. In brainstem, although there

Table 5. Radioactive labeling of brain gangliosides of cortisol-treated vs control rats*

Spot	Proportion of total dis./min (per cent control) Age (days)				
	2	6	10	15	20
Whole brain					
# 1	116	293†	144	109	126
# 2 (G_Q)	82	102	75	106	69‡
# 3 (G_{T_1})	156‡	107	88	65§	81
# 4 ($G_{D_{1b}}$)	105	86	94	52†	87
# 5	133	150	123	70	147
# 6 ($G_{D_{1a}}$)	111	87	94	126‡	93
# 7 (G_{N_1})	56†	94	197†	268†	192†
Cerebral hemispheres					
# 1	×	330†	180‖	×	135
# 2 (G_Q)	×	86	67	×	51§
# 3 (G_{T_1})	×	100	87	×	67§
# 4 ($G_{D_{1b}}$)	×	100	104	×	96
# 5	×	219†	96	×	86†
# 6 ($G_{D_{1a}}$)	×	92	98	×	110
# 7 (G_{M_1})	×	88	202‖	×	176‖
Brainstem					
# 1	×	96	152‡	×	333†
# 2 (G_Q)	×	205§	66‡	×	96
# 3 (G_{T_1})	×	104	88	×	78‡
# 4 ($G_{D_{1b}}$)	×	82‖	86	×	78
# 5	×	100	114	×	115
# 6 ($G_{D_{1a}}$)	×	91	103	×	94
# 7 (G_{M_1})	×	102	161	×	366†
Cerebellum					
# 1	×	235†	214‡	×	167
# 2 (G_Q)	×	54†	96	×	50†
# 3 (G_{T_1})	×	135‡	84	×	81
# 4 ($G_{D_{1b}}$)	×	39†	98	×	97
# 5	×	74	186‡	×	65‡
# 6 ($G_{D_{1a}}$)	×	119	100	×	106
# 7 (G_{M_1})	×	99	122	×	246†

* Experimental animals received 500 μg cortisol i.p. at 1 day of age. Each value represents six to twelve separate assays.
† $P < 0.001$.
‡ $P < 0.05$.
§ $P < 0.005$.
‖ $P < 0.01$.

was no difference overall from control, the proportion of spot # 7 was still elevated, although not as much as at 6 days. The severe deficit in cerebellar ganglioside NANA at 10 days significantly affected every spot except # 1, which contained significantly more than its control proportion of the NANA.

At 15 days of age, although there was only a non-significant decrease in whole brain total sialic acid, the NANA of spots # 1, and 5 was decreased significantly, while spots # 6 and 7 were increased. The whole brain findings at 20 days of age were the same as 15 days. These applied also when cerebral hemispheres were considered separately, although statistical significance was not always achieved. In the brainstem, the only significant differences from controls were the decrease in spot # 4 sialic acid and a decreased percentage of the total NANA in spot # 3, while the increased proportion of NANA in spot # 7 was no longer statistically significant. The decrease in spot # 2 of whole brain was accounted for largely by cerebellum, which also manifested the decrease in spot # 4 and the increases in spots # 6 and 7. In terms of the percentage distribution of NANA, only these last two alterations were significant.

The distribution of radioactive label among the seven thin-layer chromatographic spots was investigated and the results are reported in Table 5. At 2 days of age, the decrease in incorporation was manifested primarily in spot # 7, which, when prepared from control rats at this time, evidenced its greatest incorporation of radioactivity of the various ages studied. Its percentage of the total incorporation, although still greatest at this age, was also significantly below that of control animals. On the contrary, the proportion of label in spot # 3 appeared to be increased slightly. At 6 days of age, the great increase in total 24-hr uptake of label by gangliosides was evidenced in all spots, but particularly in spot # 1. When brain regions were evaluated separately, the cerebral

hemispheres paralleled the results for whole brain, but generally showed a lesser stimulation. Brainstem showed the greatest augmentation of incorporation, with spot # 2 increased in its proportion of label and spot # 4 slightly decreased. The cerebellum increased its incorporation about as much as whole brain, with spots # 1 and 3 increasing their proportion of label at the expense of spots # 2 and 4. At 10 days of age, the total incorporation was only one-third greater than controls. Spot # 1 still accounted for a significant portion of the increase but spot # 7 now entered the picture as elevated both absolutely and proportionally. The cerebrum showed a similar pattern to whole brain and stimulation of uptake remained greatest in the brainstem. In cerebellum, increased incorporation was seen in spots # 1 and 7, and spot # 5 was also increased. At 15 days of age, when total incorporation had returned almost to normal, spots # 1 and 7 remained elevated while spot # 4 actually fell somewhat below control level. In terms of percentage of total radioactivity, this represented a shift from spots # 3 and 4 toward spots # 6 and 7. This trend was maintained at 20 days, but at that time only spot # 7 persisted in its increased incorporation, while spots # 2 and 3 had fallen below control levels. In the cerebral hemispheres, the same findings were noted, and spot # 5 was also increased. In the brainstem, the severe decrease at 20 days relative to controls was manifested in all spots except # 1 and 6. The cerebellum showed a similar response, but only spot # 7 managed to achieve a control level of incorporation. Spot # 1, although affected, was still 67 per cent of control, as compared with 20–40 per cent for the other spots.

DISCUSSION

The small literature on the effects of neonatal administration of cortisol or corticosterone suggests that this hormone retards cellular proliferation in the developing brain during the period of its administration, and that the organism subsequently may be unable to compensate for the loss [28]. This would be congruent with the well-known retardation of woundhealing caused by corticosteroids [36]. In view of the previously mentioned interest in gangliosides as possible pharmacologic receptors, it is important that this study has found the neonatal administration of cortisol to alter the developmental course of brain gangliosides. Perturbations of the gangliosides pattern indicate that the changes reflect more than a mere consequence of decreased cell number.

A rapid increase in the ganglioside content of rat brain during the first 3 weeks of extra-uterine life has been reported widely [13,15,19,20,37–39]. Data concerning the activity of enzymes important in the synthesis of gangliosides in the rat brain, and on the incorporation of radionuclide into gangliosides, generally support the hypothesis of a period of rapid synthesis during week 2 of life, with leveling off of the rate of accumulation by week 4 [40–44]. The few reports of incorporation of radionuclides into gangliosides also are in accord for [^{14}C]-glucose labels [38]. Studies of other biochemical parameters (e.g. Refs. 30 and 45) indicate that the cerebral hemisphere is the part growing most rapidly during week 1 of extra-uterine life, while the cerebellum has a significant spurt of growth about midway through week 2. It appears that the brainstem has settled to a slow rate of weight increase by the time of birth, perhaps having a period of rapid growth at some prenatal stage of development.

In the present study, the concentration of ganglioside sialic acid increased in normal brain roughly 3-fold between 7 and 15 days of age, being stable before and afterward. The magnitude of this increase is identical to that reported by Suzuki [13] and Vanier *et al.* [15], as well as by others [16–18]. The period of rapid increase is in keeping with the data of Suzuki [13], Maker and Hauser [38] and Rosenberg and Stern [20], but differs from that of Vanier *et al.* [15], who reported a more uniform rate of accumulation. The results suggest that gangliosides of the brainstem develop earlier than those of the cerebral hemispheres, but reach an earlier and lower plateau. This is consistent with the greater structural and functional maturity of the brainstem at birth in the rat [27, 45], and duplicates the findings of Merat and Dickerson [19] and of Vanier *et al.* [15] for cerebrum. By 20 days of age, there was no significant difference in ganglioside concentration among the three brain parts studied, although there was a tendency toward higher values in the cerebrum.

There are clearly a rapid rise in incorporation of label from [^{14}C] glucosamine into gangliosides of rat brain up to 10 days of age and a rapid fall after that. This finding supports the argument for a critical period in ganglioside synthesis between 6 and 15 days of age, and is in agreement with previous reports [38]. In normally developing animals, the gangliosides of the cerebrum peaked in labeling at 10 days of age, while the brainstem reached its maximum at 6 days, and cerebellum rose steadily in incorporation up to 20 days. The study of incorporation of a radionuclide conveys information not obtained from data on endogenous ganglioside levels alone. The distribution of radioactivity among the brain parts varies with age, and is often radically different from that of total sialic acid content or weight. While this undoubtedly is related in part to the physical–chemical compartmentalization of the brain, it may also reveal differences in the metabolic activity of the labeled compounds.

The present study has shown that a single injection cortisol, 500 μg i.p. at 1 day of age, which is insufficient to influence overall growth characteristics permanently, affects the developmental course of the gangliosides. Cortisol-treated animals showed a diminution in weight gain and an initial retardation in brain growth, but these changes were not of great magnitude and were of variable significance when the three regions were considered separately. Not cerebrum, brainstem or cerebellum differed from controls by 20 days of age. Evaluation of the effects of hormones or drugs on the brain gangliosides is complicated by several factors. First, it has been shown that the presence of other substances, particularly basic proteins and histones, can decrease the extractability of gangliosides from brain tissue. The effect of cortisol on the endogenous levels of

these substances is unknown. Second, the presence of non-ganglioside materials in the preparation may influence the chromatographic properties of gangliosides, thus suggesting spurious hormonal effects on the ganglioside pattern. With due respect to these considerations, the cortisol treatment appears to have had a small but consistent effect on brain gangliosides. Although the decrease in brain NANA content was significantly only once each per brain and per gram wet weight, values were consistently below controls at all times after 1 week of age. The delay of 1 week before manifestation of a drug effect is contrary to the findings of others with regard to cell proliferation, where the decrease was greatest during the period of drug treatment [27]. The effect of cortisol on the incorporation of the radionuclide into total gangliosides bears an interesting relationship to the brain concentration of NANA. Incorporation is first depressed (at 2 days of age) but is then greatly stimulated (at 6 and 10 days). As the NANA content increases to control level during week 3 of life, the incorporation of radioactivity decreases also to reach control level by 20 days of age. It may be speculated that cortisol has an acute effect of inhibiting ganglioside synthesis, but also sets in motion a process which continues to deplete the gangliosides even after the effect of the drug on metabolism has ceased or has been compensated. Thus, between 2 and 6 days of age, the synthetic mechanism becomes highly active in an apparent attempt to compensate the diminished ganglioside level, but this results only in increased turnover, as indicated by the continued subnormal NANA concentration. During week 3, this effect of cortisol diminishes, allowing homeostasis to be achieved.

The brain regions differed in the responses of their gangliosides to cortisol. Ganglioside NANA was most decreased in cerebral hemispheres at 14–16 days of age, which corresponds to the period of most rapid formation of synapses identified by electron microscopy [46]. The brainstem was affected most at 6 days, the earliest time studied, in keeping with its earlier maturation. The cerebellum evinced a severe reduction in ganglioside NANA at 10 and 11 days of age, a period of rapid cellular proliferation [45]. These regional differences were apparent on the basis of tissue weight and, therefore, at least not due completely to the cortisol-induced reduction in cell number [26]. It seems, then, that cortisol had the effect of retarding ganglioside accumulation in brain particularly at periods of active cellular replication or of extensive process formation. The drug effect on incorporation of label into gangliosides was manifest in all three brain parts, but the cerebrum was affected least. Although brainstem showed the greatest compensatory increase in labeling, both brainstem and cerebrum dropped to about half the control level of incorporation at 20 days. It would be interesting to learn whether this represents a permanent alteration of ganglioside metabolism by cortisol or it is only a phase in the re-establishment of metabolic homeostasis. The neonatal administration of cortisol affected the individual gangliosides differently and changed the distribution of NANA among them relative to control animals. It is of general note that these effects seemed to be noted in the relatively most polar spot (# 1) first and to persist in the relatively least polar spot (# 7, G_{M_1}) longest. Regional differences in the effects of cortisol on individual gangliosides generally were consonant with the drug effects on total gangliosides discussed earlier.

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