The Biosynthesis of Brain Gangliosides

GANGLIOSIDE-GLYCOSYLATING ACTIVITY IN RAT BRAIN NEURONAL PERIKARYA FRACTION

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(Received 7 November 1977)

Rat brain homogenate and the synaptosomal and neuronal perikarya fractions from 17-day-old rats were compared for their activities in sialosylating endogenous gangliosides and transferring *N*-acetylneuraminic acid and galactose to several glycolipids *in vitro*. The sialosylation of endogenous gangliosides and the activities of sialosyltransferases acting either on lactosylceramide or haematoside as acceptors, as well as galactosyltransferase acting on Tay–Sachs ganglioside as acceptor, were between 3- and 12-fold higher in the neuronal perikarya fraction than in whole homogenate on a protein or ganglioside basis. The activities found in the synaptosomal fraction were negligible. No evidence was found to indicate that the low activities in this fraction were due to the presence of inhibitors of the transfer activities or to inaccessibility of the substrates to their respective enzymes. These findings, and the time course of labelling of gangliosides of the neuronal perikarya and synaptosomes from rats that received an injection of *N*-[³H]acetylmannosamine, indicate that the main cellular site of glycosylation of neuronal gangliosides is in the neuronal perikarya.

The oligosaccharide chain of the gangliosides is synthesized *in vitro* by the stepwise addition of single glycosidic groups to the corresponding lipid or glycolipid acceptor (Arce *et al.*, 1966, 1971; Kaufman *et al.*, 1966; Roseman, 1970; Maccioni *et al.*, 1972*a*). Only minor discrepancies persist with regard to the order of addition of glycosyl groups at the branching points of the chain (for reviews see Caputto *et al.*, 1974, 1976). However, the stepwise addition of sugars to gangliosides in a single pool could not explain results of incorporation *in vivo* of those glycosidic components (Maccioni *et al.*, 1971, 1972*b*). Two pools of gangliosides were postulated, one supposed to be the small pool of intermediates of the glycosylating system and the other, a much larger pool, that because it was not involved in further synthesis was designated pool of end products. This was concluded because neither precursor-to-product relationships in the labelling of the different gangliosides nor differences in the specific activities of the repeating units (galactose or sialic acid) of the carbohydrate chain were found in experiments *in vivo*. The unavailability of the end-product pool for further synthesis also implied that partial turnover of the chain, if it occurs, is a minor event (Maccioni *et al.*, 1971, 1977).

In agreement with this notion it was shown that the bulk of the microsomal gangliosides was attacked by *Clostridium perfringens* neuraminidase and converted into monosialoganglioside, but membranes desialosylated *in vitro* by treatment with this enzyme did not show any increase in the endogenous gangliosidic acceptor capacity. The pool of intermediate gangliosides was protected from the action of this neuraminidase (Maccioni *et al.*, 1974). The presence of different pools was confirmed by separation of membranes enriched in one of them: whereas the main synaptosomal fraction with high ganglioside content was very low in sialosylating activity, some membrane fractions of undetermined origin were with high sialosylating activity in relation to their ganglioside content (Landa *et al.*, 1977). The time curves of labelling *in vivo* of the gangliosides of different membranes were consistent with the possibility that the gangliosides of membranes with high sialosylating activities were precursors of the synaptosomal gangliosides (Maccioni *et al.*, 1977; Landa *et al.*, 1977).

Those results raised the question of the site of ganglioside glycosylation in the neuron, and the present work was designed to determine whether the neuronal perikarya is a site of high ganglioside-glycosylating activity.

Materials and Methods

Chemicals

Lactosylceramide (Gal β 1 $\rightarrow$ 4Glc β 1 $\rightarrow$ 1Cer)* was prepared from calf brain gangliosides (Cumar *et al.*, 1968), haematoside (NeuAc α 2 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4Glc β 1 $\rightarrow$ 1Cer) from canine erythrocytes (Yamakawa *et al.*, 1960) and Tay-Sachs ganglioside [GalNAc β 1 $\rightarrow$ 4Gal(3 $\leftarrow$ 2 α NeuAc) β 1 $\rightarrow$ 4Glc β 1 $\rightarrow$ 1Cer] from the brain of a patient with Tay-Sachs disease (Maccioni *et al.*, 1971). UDP-[14 C]galactose (200 μ Ci/ μ mol) was from the Instituto de Investigaciones Bioquímicas 'Fundación Campomar', Buenos Aires, Argentina. Poly(vinylpyrrolidone) (average mol.wt. 40000) and bovine serum albumin (fraction V) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). CMP-[3 H]NeuAc and N-[3 H]acetylmannosamine were prepared as previously described (Arce *et al.*, 1966, 1971).

Fractionation of brain tissue

Brains from 17-day-old rats were used. The synaptosomes were prepared from a washed crude mitochondrial fraction as already described (Whittaker, 1969; Landa *et al.*, 1977), but starting from brain-cortex homogenates. Neuronal perikarya were prepared by the method of Sellinger *et al.* (1971). They were washed by gentle homogenization in 0.32M-sucrose and centrifugation at 75000g for 45 min to eliminate most of the bovine serum albumin that otherwise would interfere with the protein determinations. The washed pellet was homogenized in 0.32M-sucrose or 0.2M-cacodylate/HCl buffer, pH 7.3, for determination of sialosyl- or galactosyl-transferase activities respectively. The yield of neuronal perikarya fraction was 1.77 ± 0.4 mg of protein/g of brain cortex (mean of seven determinations $\pm$ S.E.M.). This value agrees with the yield (1.63 ± 0.15 mg of protein/g of brain cortex) reported by Johnson & Sellinger (1971) for 18-day-old rats. The amount of protein obtained in the synaptosomal fraction was 0.98 ± 0.07 mg/g of brain cortex (mean of nine determinations $\pm$ S.E.M.). The integrity of the synaptosomes was verified by the ratio of occluded to total lactate dehydrogenase (see under sialosyl-transferase activity).

Morphological examination of neuronal perikarya and synaptosomes

The neuronal perikarya fraction was controlled by phase-contrast microscopy before the washing step. They appeared devoid of processes and glial cells, but low amounts of cellular detritus were often seen (Plate 1a). The synaptosomal fraction was examined

by electron microscopy. The crude mitochondrial fraction obtained at 10000g for 20 min was washed thrice and centrifuged in a sucrose gradient (Whittaker, 1969). The material floating at the interphase between 0.8M- and 1.2M-sucrose (fraction P₂B) was fixed in osmium tetroxide, dehydrated in graded ethanol and acetone and embedded in Araldite. Sections were cut in a Porter Blum ultramicrotome and stained with lead citrate and uranyl acetate (Reynolds, 1963). Plate 1(b) shows an electron micrograph of this fraction, which appears rich in synaptosomes enclosing mitochondria and synaptic vesicles. Some free mitochondria and membrane profiles are also seen.

Measurement of ganglioside-glycosylating system

Endogenous gangliosidic acceptor capacity for NeuAc was determined in an incubation system that contained, in a final volume of 25 μ l, 0.16M-sucrose, 8 μ M-CMP-[3 H]NeuAc (8×10^8 c.p.m./ μ mol), 5 mM-MnCl₂, 25 mM-cacodylate/HCl buffer, pH 6.8, and enzyme preparation (100 μ g of protein) (Arce *et al.*, 1971; Landa *et al.*, 1977). Sialosyltransferase activity with lactosylceramide as acceptor (Arce *et al.*, 1966) was measured in an incubation system that contained, in a final volume of 25 μ l, 0.16M-sucrose, 20 μ M-CMP-[3 H]NeuAc (8×10^8 c.p.m./ μ mol), 80 μ M-lactosylceramide, 15 mM-MnCl₂, 25 mM-cacodylate/HCl buffer, pH 6.8, enzyme preparation (100 μ g of protein) and 50 μ g of a mixture of the detergents Triton CF-54 and Tween 80 (2:1, w/w). In some experiments haematoside substituted for lactosylceramide in otherwise identical conditions. Galactosyltransferase was measured by the method of Basu *et al.* (1965) with Tay-Sachs ganglioside as acceptor. Labelling of gangliosides *in vivo*, radioactivity measurements and chemical determinations were carried out as previously described (Maccioni *et al.*, 1974; Landa *et al.*, 1977).

Results

Ganglioside concentration in rat brain homogenate and in synaptosomal and neuronal perikarya fractions

The ganglioside concentration in neuronal perikarya per unit weight of protein was about 2.5- and 4-fold lower than in whole homogenate and synaptosomes respectively. A survey of the literature showed that the ratios of ganglioside concentration in homogenate to neuronal perikarya and synaptosomes to neuronal perikarya varied between 4 and 5 in different species (Table 1). It is not known whether the lower concentration of gangliosides in neuronal perikarya than in synaptosomes reflects lower concentration of gangliosides in the membranes of the perikarya or lower content of membranes per mg of protein in perikarya than in synaptosomes.

* The structures of glycolipids have been abbreviated according to the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature [*Biochem. J.* (1978) 171, 21-35].

EXPLANATION OF PLATE I

Phase-contrast micrograph of the neuronal perikarya fraction (a) and electron micrograph of the synaptosomal fraction (b)
(a) The neuronal perikarya fraction was photographed through a Zeiss phase-contrast microscope. (b) The synaptosomal fraction was photographed through a Siemens Elmiskop I electron microscope.

Table 1. *Ganglioside concentration in homogenate and synaptosomal and neuronal perikarya fractions*

Values are given in nmol of gangliosidic NeuAc/mg of protein. To unify the expressions some of the values were calculated from the data provided in the references given. Values from this work are means $\pm$ S.E.M. of the numbers of determinations given in parentheses.

Brain source	Homogenate	Neuronal perikarya	Synaptosomes	Reference
Rat	18.2	3.76	—	Norton & Poduslo (1971)
Rabbit	—	9.40	49.2	Hamberger & Svennerholm (1971)
Pig	32.5	7.72	—	Tamai <i>et al.</i> (1971)
Ox	—	5.87	24.8*	Derry & Wolfe (1967)
Rat	14.7 $\pm$ 0.47 (7)	6.6 $\pm$ 0.54 (3)	24.5 $\pm$ 1.9 (5)	Present work

* Value for the whole neuropil, not for isolated synaptosomes.

Fig. 1. *Time course of the sialosylation of endogenous gangliosidic acceptors*

Incubation and determination of the incorporation into endogenous gangliosidic acceptors were carried out as described in the Materials and Methods section. ○, Neuronal perikarya fraction; ●, whole homogenate.

Fig. 2. *Protein concentration curve for sialosylation of endogenous gangliosidic acceptors*

Incubation and determination of the incorporation into endogenous gangliosidic acceptors were carried out as described in the Materials and Methods section. ○, Neuronal perikarya fraction; ●, whole homogenate; Δ, synaptosomes.

Ganglioside-glycosylating activity in whole homogenate and in synaptosomal and neuronal perikarya fractions

(a) *Sialosylation of endogenous ganglioside acceptors.* The transfer of [^3H]NeuAc from CMP-[^3H]NeuAc to endogenous gangliosidic acceptors per mg of protein was, in different experiments, between 3- and 7-fold higher in the neuronal perikarya fraction than in homogenate. Fig. 1 shows an experiment in which the rate and the total incorporation per mg of protein in the neuronal perikarya fraction was about 3-fold higher than in homogenate. The plateau reached at 45 min measured the endogenous gangliosidic acceptor capacity per mg of protein of each preparation (Arce *et al.*, 1971; Maccioni *et al.*, 1974). The incorporations into the endogenous gangliosidic

acceptors of homogenate and of neuronal perikarya and synaptosomal fractions were proportional to the amounts of preparations added when these varied from 75 to 300 μg of protein (Fig. 2).

(b) *Sialosyltransferase activity.* Determinations of detergent/protein ratios for maximal rate of transfer of sialosyl groups to exogenous acceptors by whole homogenate and neuronal perikarya fraction were carried out. The endogenous incorporation was maximal when no detergent was added (Fig. 3a), whereas incorporations into exogenous lactosylceramide (Fig. 3b) and haematoside (Fig. 3c) were very low in the absence of detergent and increased to a maximum at detergent/protein ratios ranging from 0.25 to 0.5 (w/w). At higher detergent/protein ratios a

Fig. 3. Effect of detergent on the sialosylation of endogenous and exogenous acceptors

The conditions for the sialosylation of endogenous gangliosidic acceptors (a), exogenous lactosylceramide (b) and exogenous haematoside (c) were as described in the Materials and Methods section, except for the detergent (Triton CF-54/Tween 80, 2:1, w/w)/protein ratios, which were varied as indicated. ○, Neuronal perikarya fraction; ●, whole homogenate. In (b) and (c) the endogenous incorporation was subtracted.

decrease in the transfer occurred. The transfer to exogenous haematoside was linear for amounts of fractions added from 50 to 200 μg of protein (Fig. 4). The progress of the reaction as a function of time is shown in Fig. 5.

Fig. 4. Protein concentration curve for sialosylation of exogenous haematoside

Incubation and determination of the incorporation into exogenous acceptors were carried out as described in the Materials and Methods section. ○, Neuronal perikarya fraction; ●, whole homogenate; △, synaptosomal fraction.

Fig. 5. Time course of the sialosylation of exogenous haematoside

Incubation and determination of the incorporation were as described under the Materials and Methods section. ○, Neuronal perikarya fraction; ●, whole homogenate.

The ganglioside-sialosylating activity in synaptosomes was previously found to be very low compared with that in other fractions, including the whole homogenate (Landa *et al.*, 1977). Since the synaptosomes used in previous work were obtained from homogenates prepared in 0.32M-sucrose, whereas the neuronal perikarya fraction used in this work was obtained from brain cortices minced in a solution of poly(vinylpyrrolidone), CaCl₂ and bovine serum albumin, the sialosylating activities of homogenates prepared in 0.32M-sucrose or in poly(vinylpyrrolidone), CaCl₂ and bovine serum albumin solution were compared. Table 2 shows that the incorporation into endogenous gangliosidic acceptors as well as into exogenous acceptors were almost identical in the two differently prepared homogenates. The sialosyltransferase activity per mg of protein was about 4-fold higher in neuronal perikarya than in the homogenate. On a ganglioside basis the amount of NeuAc transferred to endogenous or exogenous gangliosidic acceptors were about 10-fold higher in the neuronal perikarya than in the homogenate. Table 2 also shows that on a protein or ganglioside

basis the activity in the synaptosomal fraction was very low compared with that in whole homogenate. This is also shown for different amounts of protein in Fig. 2 and Fig. 4. Consequently, it was considered improbable that the differences in activities in the neuronal perikarya fraction and in the synaptosomal fraction were due to differences in the medium of preparation. The low activity in synaptosomes was not due to any inhibitor in the preparation, since in different experiments mixtures of synaptosomes and neuronal perikarya resulted in total activity that agreed within a variation of $\pm 20\%$ with the values expected from the addition of the activities in both fractions. Also, inaccessibility of substrates to enzymes was not the factor determining the low activity in synaptosomes, since disrupted synaptosomes did not increase the incorporation into either endogenous or exogenous acceptors. The effect of disrupting treatments on the integrity of synaptosomes was assessed by measuring the release of occluded lactate dehydrogenase (Wonnacott & Marchbanks, 1976). The activity of this enzyme measured by the method of Johnson (1960) was 99.8,

Table 2. *Endogenous gangliosidic acceptors of sialosyl groups and sialosyltransferase activity in whole homogenate and synaptosomal and neuronal perikarya fractions*

Homogenates in sucrose and in poly(vinylpyrrolidone), CaCl₂ and bovine serum albumin were obtained by homogenization of brain cortices in 10 vol. of 0.32M-sucrose or 8 vol. of a solution containing 7.5% poly(vinylpyrrolidone), 10mM-CaCl₂ and 1% bovine serum albumin respectively (PVP medium). Preparation of brain fractions, incubation conditions for the determination of endogenous gangliosidic acceptor capacity and sialosyltransferase activities and the measurement of the incorporation of sialosyl groups were as described in the Materials and Methods section. Values of sialosyltransferase activities are those in excess of the endogenous incorporation in the presence of detergent alone. Values of endogenous gangliosidic acceptor capacity are those obtained in the absence of detergent and acceptor glycolipid. The variability recorded is the S.E.M. for three determinations.

Fraction	Endogenous gangliosidic acceptor capacity		Sialosyltransferase activity: [³ H]NeuAc transfer to			
			Haematoside		Lactosylceramide	
	(c.p.m./mg of protein)	(c.p.m./nmol of NeuAc)*	(c.p.m./mg of protein)	(c.p.m./nmol of NeuAc)*	(c.p.m./mg of protein)	(c.p.m./nmol of NeuAc)*
Homogenate prepared in:						
PVP medium	4245 $\pm$ 388	288	27855 $\pm$ 1918	1896	9223 $\pm$ 1403	627
Sucrose	3985 $\pm$ 392	271	23287 $\pm$ 1756	1584	10045 $\pm$ 1294	683
Neuronal perikarya	14275 $\pm$ 1554	2166	91553 $\pm$ 5527	13892	45789 $\pm$ 2736	6948
Synaptosomes	314 $\pm$ 56	12.8	—	—	—	—
Synaptosomes disrupted by:						
Hypo-osmotic shock†	350					
Sonication‡	360					
Addition of detergent§	276		3443 $\pm$ 1515	140	1533	62

* The value of ganglioside concentration used for this ratio is the corresponding mean of our determinations shown in Table 1.

† The synaptosomal preparation was suspended in water to give a solution of approx. 0.08M-sucrose, maintained for 1h at 4°C and then assayed.

‡ The synaptosomal preparation was sonicated in an iced-water bath during two 15s periods separated by a 60s interval. A Branson model B12 Sonifier was used.

§ The synaptosomal preparation was mixed with a solution of Triton CF-54/Tween 80 (2:1, w/w) to give a detergent/protein ratio of 0.5 (w/w).

Fig. 6. Time course of labelling of gangliosides of the synaptosomal and neuronal perikarya fractions from rats injected with N -[^3H]acetylmannosamine

Rats (17-day-old) were injected with $30\mu\text{Ci}$ of N -[^3H]acetylmannosamine (200mCi/mmol) intracisternally and returned to their mothers. At each of the indicated times two rats were killed by decapitation and the brain cortices were removed in the cold. A pool was made with the right hemicortex of one rat and the left hemicortex of the other for isolation of the synaptosomal fraction and another pool with the two remainder hemicortices for isolation of the

397, 967 and 657nmol of NADH_2 oxidized/min per mg of protein in intact, hypo-osmotically shocked, sonicated and detergent-treated synaptosomes respectively.

(c) *Galactosyltransferase activity*. This activity was assayed in whole homogenate, in neuronal perikarya fraction and in the synaptosomal fraction with Tay-Sachs ganglioside as acceptor. The specific galactosyltransferase activity was about 5-fold higher in the neuronal perikarya fraction than in homogenate and was very low in the synaptosomal fraction. The values obtained for mixtures of synaptosomal and neuronal perikarya fractions were those expected for mixtures of uninhibited samples (Table 3).

According to the two-pools hypothesis the bulk of gangliosides are end products unavailable for the synthesis of gangliosides of higher complexity, whereas the endogenous gangliosidic acceptors together with the cognate glycosyltransferase activities are commensurate with the small pool of intermediates (Caputto *et al.*, 1976; Maccioni *et al.*, 1977). Consequently, from the results shown above, it appears that the synaptosomal and the neuronal perikarya fractions are enriched in the pool of end products and intermediate gangliosides respectively.

Studies in vivo

The time course of incorporation *in vivo* of radioactivity from N -[^3H]acetylmannosamine into gangliosides was followed in the neuronal perikarya and nerve-ending fractions. Fig. 6(a) shows that during

neuronal perikarya fraction. Procedures for the preparation of the fractions were as indicated in the Materials and Methods section. $\circ$, Gangliosides from the neuronal perikarya fraction; $\bullet$, gangliosides from the synaptosomal fraction. Δ , labelling ratio. (a) Expt. 1; (b) Expt. 2.

Table 3. *Galactosyltransferase activity in homogenate and synaptosomal and neuronal perikarya fractions*

Fractions were prepared as indicated in the Materials and Methods section. The incubation system for the galactosyltransferase assay was that described by Basu *et al.* (1965). The incorporation of galactosyl groups was measured by following the same methodology as for the measurement of the incorporation of sialosyl groups (Maccioni *et al.*, 1974; Landa *et al.*, 1977). Values of incorporation into Tay-Sachs ganglioside are those in excess of the endogenous incorporation. Values are means $\pm$ S.E.M. for the numbers of determinations indicated in parentheses.

Fraction	Amount in the assay (μg of protein)	Acceptor ...	Radioactivity incorporated (c.p.m.)	
			None	Tay-Sachs ganglioside
Homogenate	400		82 ± 26 (3)	318 ± 71 (4)
Neuronal perikarya	400		140 ± 10 (3)	1569 ± 150 (5)
Synaptosomes	400		23 ± 3 (3)	60 ± 20 (3)
Mixture of:				
Neuronal perikarya	200		92 ± 32 (3)	884 ± 63 (3)
Synaptosomes	200			

the 4–8 h following a single injection of the precursor the specific radioactivity of the neuronal perikarya gangliosides was higher than that of the synaptosomal gangliosides and after this period they tended to equilibrate. A lag period in the labelling of synaptosomal gangliosides was noted during 2 h after the injection (Fig. 6b). The ratio between the specific radioactivities of the gangliosides from the neuronal perikarya and those from the synaptosomal fraction decreased from 6–7 in the first 2 h to approx. 1 after 8 h.

Discussion

During a search for the localization of the pool of intermediate gangliosides (for reviews see Caputto *et al.*, 1976; Maccioni *et al.*, 1977) it was found that rat brain synaptosomes obtained from a washed crude mitochondrial fraction were lower than other membrane fractions in ganglioside-sialosylating activity *in vitro* and in the labelling at short times after injection to the rat of radioactive precursors (Landa *et al.*, 1977). In the present work it is shown that the ganglioside-glycosylating activity in the neuronal perikarya fraction is higher than in the whole homogenate and in synaptosomes when compared on a protein or ganglioside basis. The values for endogenous acceptor capacity and for sialosyltransferase or galactosyltransferase activities in this later subcellular fraction are so low with respect to the same values in the neuronal perikarya fraction that the possibility that these activities in the synaptosomes were due to contaminating membranes originated in destroyed neuronal perikarya cannot be discarded at present. The time course of the labelling *in vivo* of the gangliosides in the synaptosomal and neuronal perikarya fractions is compatible with the assumption that gangliosides glycosylated in the neuronal perikarya were the origin of the synaptosomal gangliosides. In cortices of 17-day-old rats that received a pulse of *N*-[³H]acetylmannosamine the labelling of the gangliosides in the neuronal perikarya reached a peak at about 4–5 h after the injection and then decreased, whereas in the synaptosomes the labelling of the gangliosides increased up to 15 h, when it reached the values in the perikarya.

The subcellular distribution of the ganglioside-glycosylating enzymes in brain is uncertain at present. It has been claimed that they are localized in the synaptosomal fraction (Roseman, 1970; Di Cesare & Dain, 1972; Den *et al.*, 1975) although the glucosyltransferase acting on the synthesis of glucocerebrosides was found mainly in the microsomal fraction (Shah, 1973; Neskovic *et al.*, 1973). Jones *et al.* (1972) have shown that the neuronal perikarya are able to label their own gangliosides when incubated with radioactive serine and NeuAc, whereas Radin *et al.* (1972) showed the presence in this fraction of enzymes

that catalyse the transfer of glucose from UDP-glucose to ceramide to form glucosylceramide. On the other hand, Den *et al.* (1975) could not find any enrichment of the UDP-galactose–(Tay–Sachs ganglioside) galactosyltransferase in the neuronal perikarya over other fractions of the embryonic chicken brain.

The results of the present work together with those reported previously (Landa *et al.*, 1977), although not disproving conclusively that the synaptosomes can glycosylate gangliosides, do point to the neuronal perikarya as the main site for the synthesis of gangliosides, whereas the synaptosomes appear as the main structure containing completed gangliosides. It will eventually be necessary to investigate which of the components of the perikarya fraction is responsible for the glycosylating activities. In view of current claims synaptosomes attached to the neuronal body should be investigated for the possibility that they may show activities that are not present in the bulk of the synaptosomes isolated by conventional methods. The other component that deserves attention is the Golgi apparatus. This is the main glycosylating structure in other cells (Keenan *et al.*, 1974) and, if this were the case for neurons, glycosylation of glycoconjugates in internal membrane before the translocation to the plasma membrane (Hirano *et al.*, 1972) would also be applicable to the biosynthesis of neuronal gangliosides. The controversial issue of the axonal transport of gangliosides (Forman & Ledeen, 1972; Holm, 1972; Rösner *et al.*, 1973; Rösner, 1975; Maccioni *et al.*, 1977) appears to have further reasons to be investigated until current ambiguities are resolved.

We are indebted to Dr. E. Hliba for performing the electron-microscopic studies and to Dr. A. Arce for helpful discussions. This investigation was supported in part by a grant from the Consejo Nacional de Investigaciones Científicas y Técnicas, by a grant from the Organization of American States and by a grant from the Fundación 'Lucio Cherny'.

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