Ganglioside Synthesis during the Development of Neuronal Polarity

Major Changes Occur during Axonogenesis and Axon Elongation, but Not during Dendrite Growth or Synaptogenesis*

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Changes in the levels and types of gangliosides occur during neuronal differentiation and development, but no studies have correlated these changes with defined events in neuronal morphogenesis. Here, we have analyzed the relationship between ganglioside synthesis and the development of axons and dendrites in polarized neurons, using hippocampal neurons cultured in such a way that axons and dendrites are generated by a defined sequence of events and in which there is virtually no contamination by glial cells. Neurons were analyzed by addition of N-acetylgalactosamine to GM1, the first ganglioside of the a-series. Gangliosides GM1, GM3 and GM4 are referred to as “simple” gangliosides, and all subsequent gangliosides in the metabolic pathway as “complex” gangliosides (Fig. 1). Ganglioside synthesis can be studied using various precursors, including radioactive sphingoid bases such as 4,5-<sup>3</sup>H)dihydrosphingosine (5, 8, 9).

Many studies have demonstrated that ganglioside levels and types change during neuronal differentiation and development (see, for example, Refs. 10–18). However, the ability to correlate ganglioside synthesis with a specific developmental event has been limited by the use of neuronal cell lines, in which the main developmental event is transformation of a non-neuronal to a neuronal phenotype (i.e. formation of a neurite), or of primary cultures, in which neuronal development is not well characterized and in which the contribution of glia to ganglioside synthesis is difficult to distinguish from that of neurons. Ganglioside synthesis has also been analyzed in developing brain (13), but similar problems of characterization and glial contamination exist.

Here, we have analyzed ganglioside synthesis in hippocampal neurons cultured in such a way that axons and dendrites develop by a known sequence of events and can be distinguished both morphologically and biochemically (19). Hippocampal neurons have been extensively characterized when cultured at low densities (19–25). Cells are cultured from the hippocampus of embryonic day 18 rats (20); at this time, the hippocampus is inhabited mainly by pyramidal neurons that are at the transition stage of withdrawal from proliferation and at the beginning of differentiation (26). In the initial stages of growth (stages 1 and 2; see Ref. 19), each neuron develops a number of short processes, and after some hours, one of the processes starts to grow rapidly. The rapidly growing process develops axonal characteristics (stage 3) and can be distinguished from the minor processes by the presence of proteins such as GAP-43 (22) and synaptophysin (23). During the next stage of growth (stage 4), minor processes begin to elongate.

The sialic acid-containing glycosphingolipids (GSLs), the gangliosides, are found at high levels in the plasma membrane (1) of neuronal tissues and may play important roles in neuronal function (2, 3). GSL synthesis begins at the cytosolic surface of the endoplasmic reticulum, with formation of the sphingoid long chain base (4) and of dihydroceramide (4, 5), and is completed in the Golgi apparatus, where most of the glycosylation reactions occur (6, 7). The first ganglioside synthesized is GM₃ (2), formed by sialylation of lactosylceramide (Fig. 1). GM₃ can be sialylated to G₂₃, the first ganglioside of the b-series, or modified by addition of N-acetylgalactosamine to G₂₃, the first ganglioside of the a-series. Gangliosides GM₃ and G₂₃ are referred to as “simple” gangliosides, and all subsequent gangliosides in the metabolic pathway as “complex” gangliosides (Fig. 1).

 gangliosides and glycosphingolipids are named according to Svennerholm (38) and are defined as follows: LacCer; Gal[1→4]GlcCer; G₃₋₄₄, NeuAc(2→3)Gal[1→4]GlcCer; G₄₋₅₂, GalNAc[1→4]Gal[1→4]NeuAc[2→3]Gal[1→4]GlcCer; G₄₋₅₂₃, Gal[1→3]GanNAc[1→4]Gal[1→3]NeuAc[2→3]Gal[1→4]GlcCer; G₄₋₅₂₃₄, NeuAc[2→3]Gal[1→3]GanNAc[1→4]Gal[1→3]NeuAc[2→3]Gal[1→4]GlcCer; G₄₋₅₂₃₄₅, Gal[1→3]GanNAc[1→4]Gal[1→3]NeuAc[2→3]Gal[1→4]GlcCer; G₄₋₅₂₃₄₅₆, NeuAc[2→3]Gal[1→3]GanNAc[1→4]Gal[1→3]NeuAc[2→3]Gal[1→4]GlcCer; G₄₋₅₂₃₄₅₆₇, NeuAc[2→3]Gal[1→3]GanNAc[1→4]Gal[1→3]NeuAc[2→3]Gal[1→4]GlcCer; G₄₋₅₂₃₄₅₆₇₈, NeuAc[2→3]Gal[1→3]GanNAc[1→4]Gal[1→3]NeuAc[2→3]Gal[1→4]GlcCer.

1 The abbreviations used are: GSLs, glycosphingolipids; MAP-2, microtubule-associated protein-2; GlcCer, glucosylceramide; LacCer, lactosylceramide; PSL, phospholipids.
**EXPERIMENTAL PROCEDURES**

**Hippocampal Cultures**—Hippocampal neurons were cultured at high density based on methods used for low density hippocampal cultures (20). The dissected hippocampi of embryonic day 18 rats (Wistar) were dissociated by trypsinization (0.25% (w/v) for 15 min at 37°C). The dissected hippocampi of embryonic day 18 rats (Wistar) were cultured on a rubber policeman, and coverslips were washed four times with water. The suspended cells were lyophilized, and the dry material was extracted using CHCl3/CH2OH/9.8 mM CaCl2 (60:35:8, v/v/v) as the developing solvent; [4,5-3H]Dihydrosphingosine was eluted using CH3OH and separated from sphingosine by preparative TLC using CHCl3/CH2OH/NH3 (40:10:1, v/v/v) as the developing solvent. [4,5-3H]Dihydrosphingosine was stored in toluene/ethanol (95:5, v/v) at −80°C.

**Characterization of Neuronal Development—**Neuronal development was assessed by immunofluorescence using antibodies against axon-specific (GAP-43 and synaptophysin) and dendrite-specific (MAP-2) proteins (20). Glia were identified using an anti-glial fibrillary-associated protein antibody. Neurons were fixed in 4% paraformaldehyde in phosphate-buffered saline containing 4% sucrose for 20 min at 37°C, permeabilized with 0.25% Triton X-100 for 5 min at 37°C, and then incubated for 1 h at 37°C with primary antibodies (Biomaker, Kiyat Weizmann, Revhotov, Israel); anti-glial fibrillary-associated protein, anti-MAP-2, and anti-synaptophysin antibodies were diluted 1:200 in phosphate-buffered saline, and the anti-GAP-43 antibody was diluted 1:1000. A rhodamine-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) was used for detection. Cells were observed using Plan Apochromat 40×/1.3 NA or 63×/1.4 NA objectives of a Zeiss Axiovert 35 microscope with an appropriate filter for rhodamine fluorescence. Cells were photographed using a Contax 167MT camera and Eastman Kodak 400 film.

**Synthesis of [4,5-3H]Dihydrosphingosine—**[4,5-3H]Dihydrosphingosine was synthesized by reduction of d-erythro-sphingosine (Sigma) with NaBH4 (10 Ci/mmol) (5, 27). Sphingosine (10 mg) was dissolved in tetrahydrofuran/H2O (1:1, v/v), frozen in liquid N2, and overlayed with Pd(Ac)2 (5 μmol), glacial acetic acid (20 μl), and NaBH4 (100 μCi; dissolved in 1 μl NaOH). After each overlay, the reaction mixture was refrigerated. Tetrahydrofuran was passed through an activated carbon column prior to use to remove peroxides. The frozen vial was flushed with argon, sealed, and allowed to thaw at 25°C, and the reaction mixture was stirred for 24 h. The reaction was terminated by filling the vial with water, and the reaction mixture was passed over an RP-18 reverse-phase chromatography column (Merck). The column was washed with H2O until no 3H radioactivity could be detected in the eluant. [4,5-3H]Dihydrosphingosine was eluted using CH3OH and separated from sphingosine by preparative TLC using CHCl3/CH2OH/NH3 aqueous NH3OH (40:10:1, v/v/v) as the developing solvent. [4,5-3H]Dihydrosphingosine was stored in toluene/ethanol (95:5, v/v) at −80°C.

**Ganglioside Metabolism and Neuronal Polarity**

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RESULTS

Characterization of Neuronal Cultures—Hippocampal neurons cultured at low density have been well characterized (19, 20). However, due to the paucity of cellular material, low density cultures are not suitable for biochemical analysis. We have now characterized the development of hippocampal neurons cultured by a similar method but at sufficiently high densities to provide enough material for biochemical analysis of [3H]-GSL synthesis.

Within a few hours after plating, neurons extend lamellipodia, followed by the formation of minor processes (Fig. 2A). Within the next 24–48 h, one of the processes starts to grow rapidly (Fig. 2B), and within 2–3 days, a dense network of neuronal processes has formed, rendering impossible the identification of the processes as either axons or dendrites by light microscopic criteria (Fig. 2, C and D). However, by 5 (not shown) and 8 days in culture (Fig. 2, E and F), dendrites can be distinguished using an anti-MAP-2 antibody; most of the processes are not labeled using this antibody, indicating that they are axons. Likewise, after 15 days, MAP-2-positive dendrites can be distinguished from the dense axonal network (Fig. 2, G and H), and by 15 days, synaptic connections have been formed, as indicated by the localization of synaptophysin (23) to presynaptic boutons along the axon (Fig. 2, I and J). Axons were also identified using an anti-GAP-43 antibody (22).

The number of cells remaining on the coverslips at various days of culture was ascertained by counting six separate fields on two individual coverslips/day; analyses were combined from two separate neuronal cultures. On day 0, there were −110,000 cells/coverslip; day 1, 92,000 cells; day 5, 80,000 cells; day 8, 55,000 cells; and day 15, 45,000 cells.

The number of glial cells was estimated by counting the number of glial fibrillary-associated protein-positive cells. The percent of glial cells, compared with neurons, was 3.6 ± 2.5, 8.2 ± 1.0, and 7.0 ± 1.0% (mean ± S.E.; 40 microscopic fields were analyzed per coverslip/day for two individual cultures) on days 6, 8, and 15, respectively. The extremely low levels of glial contamination, and the sequence of developmental events identical to that observed in low density cultures, render high density cultures of hippocampal neurons an attractive and unique system for analyzing GSL synthesis during the development of neuronal polarity. However, the amount of protein/coverslip could not be reliably estimated (analyzed as described in Ref. 31). On days 0–5, protein levels were usually below 1 μg/coverslip, whereas on days 8 and 15, −3–5 μg of protein was detected per coverslip.

Rate of [4,5-3H]Dihydrosphingosine Uptake and [3H]-GSL Synthesis—Initial experiments were performed to characterize [4,5-3H]dihydrosphingosine uptake. Dishes containing four neuronal coverslips were incubated with 5 × 10^6 cpm of [4,5-3H]dihydrosphingosine (10 Ci/mmol) for various times. After short incubations (30 min), 337,000 cpm of [3H]radioactivity were extracted from coverslips of 2-day-old neurons, whereas 508,000 cpm were extracted from coverslips of 8-day-old neurons. Using a variety of TLC solvent systems and authentic sphingolipid standards (5, 9, 32), we determined that after 30 min of incubation, most (~60%) of the recovered cell-associated [3H]radioactivity consisted of [4,5-3H]dihydrosphingosine, with some [4,5-3H]dihydroceramide (~30%), [3H]ceramide, and [3H]phosphatidylcholine. This indicates that analysis of cell-associated [3H]radioactivity (Fig. 3A) reflects mainly [4,5-3H]dihydrosphingosine uptake, together with some “trapping” of acylated derivatives in cellular membranes. However, the amount of radioactivity in [4,5-3H]dihydrosphingosine is an underesti-
to those observed in rat microsomal membranes (5) and results from the rapid transfer and partitioning of [4,5-3H]dihydrosphingosine into membranes. The 2.21-fold difference in the amount of uptake between 2- and 8-day-old neurons is presumably due to the greater membrane surface area/cell in older neurons (see Fig. 2, C and E), which results from rapid axon and dendrite growth.

A lag period of between 30 min and 1 h was observed before significant amounts of 3H radioactivity were recovered in 3H-GSLs. However, for incubations of between 1 and 9 h, the rate of synthesis of total 3H-GSLs was linear versus time (Fig. 3B) for both 2- and 8-day-old neurons, with rates of synthesis of 182 and 452 fmol/h/10⁴ cells, respectively. The similarity between the ratios of the initial rates of [4,5-3H]dihydrosphingosine uptake (2.21) and the ratio of the rates of 3H-GSL synthesis (2.48) on days 2 and 8 suggests that the rate of 3H-GSL synthesis is proportional to the initial rate of [4,5-3H]dihydrosphingosine uptake. This is supported by observations that the rate of 3H-GSL synthesis remains linear even after longer periods of incubation (Fig. 3B), despite a significant decrease in the rate of accumulation of 3H radioactivity in neurons after 1 h (Fig. 3A).

Comparison of initial rates of synthesis (0–1 h) of individual 3H-GSLs with later rates of synthesis (1–6 h) demonstrated that there is a significant lag period in the synthesis of complex 3H-GSLs, but not of simple 3H-GSLs (Table I). For instance, the initial rate of synthesis of ganglioside [3H]GlcCer and [3H]GT1b decreased as the time of incubation increased (Table I). Moreover, the ratio of the rates of initial versus late synthesis of individual 3H-GSLs increased in a fashion consistent with the pathway of ganglioside synthesis shown in Fig. 1. Thus, the ratio of initial versus late synthesis for gangliosides [3H]GM₃ and [3H]GT₁b was significantly lower than that for [3H]GM₁ and [3H]GD₁b, respectively. Finally, the increase in the rate of synthesis of individual 3H-GSLs on day 8 compared with day 2 is consistent with the idea that the rate of 3H-GSL synthesis is a direct result of the rate of [4,5-3H]dihydrosphingosine uptake (see above).

3H-GSL Synthesis During Development of Neuronal Polarity—We next examined the synthesis of 3H-GSLs (Fig. 4) on different days of culture (Fig. 2). Neurons were incubated with 5 × 10⁶ cpm of [4,5-3H]dihydrosphingosine for 6 h; the synthesis of all 3H-GSLs was linear versus time during this period (Fig. 3 and Table I). Consistent with data obtained by analysis of the rates of total 3H-GSL synthesis on days 2 and 8 of culture (Fig. 3B), the synthesis of 3H-GSLs increased by 2.58-fold between days 2 and 8 during a 6-h incubation (Table II). Between days 2 and 8, the synthesis of 3H-GSLs increased by 12.5-fold. A particularly noticeable increase in total 3H-GSL synthesis occurred between days 0 and 1 of culture. During this period, the surface area of most neurons increased significantly due to the rapid growth of one neuronal process and of a number of short processes (Fig. 2, A and B).

The synthesis of all individual 3H-GSLs increased versus days of culture (Table II), but the amount of increase varied for individual 3H-GSLs (Table III). For instance, [3H]GM₃ synthesis increased by 4-fold between days 0 and 15, but [3H]GM₁...

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TABLE I

Rates of synthesis of 3H-GSLs in cultured hippocampal neurons

| GSL   | Initial rate (0–1 h) | Day 2 | Late rate (1–6 h) | Ratio | Day 8 | Late rate (1–6 h) | Ratio |
|-------|----------------------|-------|-------------------|-------|-------|-------------------|-------|
| GlcCer| 22.3                 | 11.9  | 0.53              |       | 69.3  | 49.1              | 0.70  |
| G₄a₂₀ | 12.9                 | 12.1  | 0.93              |       | 12.9  | 10.6              | 0.82  |
| a-series |                  |       |                   |       |       |                   |       |
| G₄₀₂₀ | 5.4                  | 11.2  | 2.07              |       | 18.2  | 43.1              | 2.36  |
| G₄₀₂₀ | 10.1                 | 29.8  | 2.95              |       | 34.6  | 97.1              | 2.84  |
| b-series |                  |       |                   |       |       |                   |       |
| C₂₀₁ | 14.0                 | 26.1  | 1.86              |       | 29.6  | 42.6              | 1.43  |
| C₂₀₁b | 13.6                 | 46.8  | 3.43              |       | 29.6  | 83.5              | 2.82  |
| C₂₀₁b | 20.6                 | 64.9  | 3.14              |       | 44.5  | 108.5             | 2.43  |
| C₂₀₁b | 6.6                  | 18.8  | 2.84              |       | 7.0   | 25.0              | 3.56  |

*Note that during 6 h of incubation, there is virtually no degradation of 3H-labeled gangliosides. Therefore, the rates obtained are those of 3H-labeled ganglioside synthesis, and not a combination of their synthesis, degradation, and further metabolism (K. Hirschberg and A. Futerman, manuscript in preparation).*

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Fig. 3. Uptake of [4,5-3H]dihydrosphingosine and rate of 3H-GSL synthesis. 2-day-old (□) or 8-day-old (■) neurons were incubated with 225 fmol of [4,5-3H]dihydrosphingosine. After various times, lipids were extracted, and the total cell-associated 3H radioactivity/10⁴ cells was measured (A). Since only 6% of the 3H radioactivity was recovered in 3H-GSLs after 1 h of incubation, the total recovered 3H radioactivity after 0, 0.5, and 1 h was used as an estimate of the rate of [4,5-3H]dihydrosphingosine uptake; linear regression analysis gave rates of uptake of 24.2 (r = 0.96) and 53.5 (r = 0.96) cpm/min/10⁴ cells for 2- and 8-day-old neurons, respectively (A). During the initial 30–60 min of incubation with [4,5-3H]dihydrosphingosine, barely detectable levels of 3H-GSL synthesis were observed (B). However, the rate of 3H-GSL synthesis was linear between 1 and 9 h of incubation for both 2- and 8-day-old neurons, with rates of 182 (r = 0.98) and 452 (r = 0.99) fmol/h/10⁴ cells, respectively.
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Fig. 4. TLC analysis of 3H-GSL synthesis during neuronal development. Neurons from different days of culture (as indicated) were incubated with 225 fmol of [4,5-3H]dihydrosphingosine for 6 h prior to extraction and separation by TLC. 3H-GSLs were identified by comparison with authentic standards. Each lane was loaded with 150,000 cpm of the extracted 3H-GSLs, which were visualized using ENHANCE spray. Note that lipids extracted from 0-8-day-old neurons were separated on a different TLC plate than 15-day-old neurons, but the same amount of 3H radioactivity was loaded onto each plate, and the same exposure times to x-ray film were used.

Table II

3H-GSL synthesis during neuronal development

| GSL | 0 day* | 1 day | 2 days | 3 days | 5 days | 8 days | 15 days |
|-----|--------|-------|--------|--------|--------|--------|---------|
| GlcCer | 50 ± 3 | 109 ± 12 | 161 ± 91 | 150 ± 54 | 144 ± 43 | 188 ± 20 | 245 ± 39 |
| LacCer | 10 ± 3 | 28 ± 7 | 26 ± 13 | 43 ± 6 | 98 ± 17 | 163 ± 25 | 172 ± 46 |
| Ga3 | 21 ± 3 | 65 ± 19 | 77 ± 31 | 89 ± 24 | 94 ± 20 | 187 ± 90 | 84 ± 10 |
| a-series | | | | | | | |
| Ga2 | 4 ± 1 | 25 ± 6 | 16 ± 4 | 48 ± 28 | 63 ± 23 | 141 ± 69 | 46 ± 1 |
| Ga1 | 11 ± 1 | 73 ± 6 | 79 ± 33 | 110 ± 28 | 93 ± 13 | 210 ± 82 | 328 ± 28 |
| Ga1a | 14 ± 1 | 96 ± 10 | 112 ± 40 | 165 ± 34 | 187 ± 22 | 360 ± 100 | 538 ± 55 |
| b-series | | | | | | | |
| Gb3 | 49 ± 4 | 91 ± 6 | 77 ± 35 | 112 ± 27 | 138 ± 22 | 216 ± 78 | 109 ± 3 |
| Gb1b | 20 ± 3 | 145 ± 16 | 103 ± 30 | 153 ± 32 | 180 ± 43 | 369 ± 71 | 455 ± 49 |
| Gb1a | 28 ± 6 | 217 ± 21 | 150 ± 35 | 162 ± 30 | 193 ± 63 | 318 ± 73 | 519 ± 63 |
| Gb1b | 6 ± 1 | 51 ± 5 | 33 ± 6 | 37 ± 6 | 42 ± 14 | 70 ± 3 | 83 ± 11 |
| Total 3H-GSL synthesis | 206 ± 22 | 900 ± 28 | 836 ± 314 | 1068 ± 213 | 1214 ± 180 | 2160 ± 490 | 2581 ± 292 |

* Day(s) in culture.

Table III

Profile of 3H-GSL synthesis during neuronal development

Data are taken from Table II and expressed as a percentage of total 3H-GSL synthesis.

| GSL | 0 day* | 1 day | 2 days | 3 days | 5 days | 8 days | 15 days |
|-----|--------|-------|--------|--------|--------|--------|---------|
| GlcCer | 24.8 ± 3.8 | 12.1 ± 1.5 | 16.9 ± 3.4 | 13.2 ± 2.2 | 11.7 ± 2.2 | 9.4 ± 1.8 | 9.4 ± 0.5 |
| LacCer | 4.6 ± 1.2 | 3.2 ± 0.9 | 3.0 ± 0.3 | 4.5 ± 0.9 | 8.2 ± 1.0 | 7.8 ± 0.7 | 6.5 ± 1.1 |
| Ga3 | 6.3 ± 3.1 | 7.3 ± 2.2 | 9.1 ± 1.3 | 8.5 ± 1.3 | 8.2 ± 2.1 | 6.7 ± 2.6 | 3.3 ± 0.1 |
| a-series | | | | | | | |
| Ga2 | 2.3 ± 0.6 | 2.8 ± 0.7 | 2.1 ± 0.4 | 4.1 ± 1.9 | 5.4 ± 1.8 | 6.0 ± 3.0 | 1.8 ± 0.2 |
| Ga1 | 5.2 ± 0.2 | 8.0 ± 0.4 | 9.2 ± 0.4 | 9.8 ± 0.6 | 7.7 ± 0.6 | 9.3 ± 1.8 | 12.8 ± 0.4 |
| Ga1a | 7.0 ± 0.2 | 10.6 ± 0.8 | 13.6 ± 0.2 | 15.3 ± 0.5 | 15.8 ± 1.5 | 16.2 ± 1.2 | 20.9 ± 0.4 |
| b-series | | | | | | | |
| Gb3 | 24.1 ± 1.0 | 10.2 ± 0.5 | 8.7 ± 0.7 | 10.7 ± 1.6 | 11.6 ± 1.8 | 9.8 ± 3.1 | 4.3 ± 0.4 |
| Gb1b | 9.5 ± 0.4 | 16.1 ± 1.6 | 13.1 ± 1.3 | 14.2 ± 1.1 | 14.4 ± 2.4 | 17.4 ± 0.6 | 17.7 ± 0.6 |
| Gb1a | 13.1 ± 1.4 | 24.2 ± 2.2 | 19.7 ± 2.0 | 16.0 ± 2.0 | 15.3 ± 3.9 | 16.0 ± 4.4 | 20.1 ± 0.4 |
| Gb1b | 3.1 ± 0.1 | 5.7 ± 0.5 | 4.6 ± 1.0 | 3.7 ± 0.5 | 3.4 ± 1.0 | 3.6 ± 0.8 | 3.2 ± 0.1 |

* Day(s) in culture.

synthesis increased by 30-fold (Table II). Analysis of the distribution of 3H radioactivity between individual 3H-GSLs (Fig. 4 and Table III) revealed three major changes in the profile of newly synthesized 3H-GSLs during the development of neuronal polarity, as follows.

(i) The ratio of simple to complex 3H-GSL synthesis changes during the first day of culture. During this period of lamellipodia (stage 1; see Ref. 19) and minor process formation (stage 2; see Fig. 2A), the predominant 3H-GSLs synthesized are [3H]GlcCer and [3H]Ga3 (Tables II and III); together with [3H]LacCer and [3H]Gm3, these simple GSLs compose 60% of the total 3H-GSLs synthesized on day 0 (Fig. 5). However, on day 1 of culture, by which time one of the minor processes has begun to grow rapidly (stage 3; see Fig. 2B), the simple GSLs compose
only 33% of the total $^3$H-GSLs synthesized (Fig. 5), with significantly higher levels of synthesis of complex gangliosides of both the a- and b-series (Table III).

(ii) The ratio of simple to complex gangliosides does not change significantly after day 1 of culture, but the ratio of a- to b-series gangliosides increases rapidly during the first 4 days. After formation of the process that is destined to become the axon (stage 3), a period of rapid axon growth continues for the next 3–4 days (Fig. 2, C and D), during which time the minor processes grow only very slowly (19). During this period, the ratio of simple to complex gangliosides does not change (Fig. 5). However, there is significant change in the ratio of complex a- to b-series gangliosides (Fig. 6). On day 1 of culture, a-series gangliosides compose 21% of the total $^3$H-GSLs synthesized, but by day 5, they compose 29% (Table III). Since there is a corresponding decrease in the synthesis of b-series gangliosides (Table III), a significant increase in the ratio of a- to b-series gangliosides is obtained (Fig. 6).

(iii) There is a small increase in the ratio of a- to b-series gangliosides from days 5 to 14, by which time the major gangliosides synthesized are $G_{0,3a}, G_{1,3a}, G_{3,1p}$, and $G_{3,1a}$. Between days 5 and 15, minor processes acquire the characteristics of dendrites (stage 4; see Fig. 2, E–H), and synaptogenesis occurs (stage 5; see Fig. 2, I and J). Despite these major morphological and functional changes, there is little alteration in the pattern of ganglioside synthesis during this period. The ratio of a- to b-series gangliosides continues to increase, but at a much slower rate than that observed between days 1 and 5 (Fig. 6). On day 8, gangliosides $G_{1,4a}, G_{5,2a}, G_{3,1b}$, and $G_{1,1b}$ compose 59% of the total $^3$H-GSLs synthesized, but by day 15, these four gangliosides compose 71% (Table III), and simple gangliosides compose only 23% of the total $^3$H-GSLs synthesized (Fig. 5).

$^3$H-GSL Synthesis in Glia—To determine whether the small number of glial cells found on neuronal coverslips (between 3 and 8%; see above) contributed significantly to $^3$H-GSL synthesis, glia were grown to near confluence and incubated with [4,5-$^3$H]dihydrosphingosine under conditions identical to those used to label neurons. After 6 h of incubation, 795 fmol of $^3$H-GSLs was synthesized, with 492 and 165 fmol in gangliosides $[^3$H]$G_{0,3a}$ and $[^3$H]$G_{1,3a}$, respectively; the remaining $^3$H radioactivity was recovered in $[^3$H]$LacCer$ and $[^3$H]$GlcCer$, and no synthesis of any complex $^3$H-GSLs was observed. The low levels of synthesis of $^3$H-GSLs, the lack of synthesis of complex $^3$H-GSLs, and the small number of glia on neuronal coverslips indicate that the contribution of glia to $^3$H-GSL synthesis on neuronal coverslips is negligible.

DISCUSSION

In this study, we have analyzed ganglioside synthesis during the development of neuronal polarity. Ganglioside synthesis was studied after relatively short times of incubation (up to 6 h), unlike many previous studies, which either analyzed total GSL content or analyzed GSL synthesis after long times of incubation (24–48 h), in which the rate of synthesis could not be accurately determined due to turnover and degradation of labeled GSLs.

Ganglioside Synthesis during Axonogenesis and Rapid Axon Growth—The most dramatic changes in ganglioside synthesis are the increase in complex ganglioside synthesis on day 1 (stages 2 and 3; see Ref. 19) compared with day 0 (stage 1) and the increase in the ratio of a- to b-series gangliosides between days 1 and 5 (stage 3). A decrease in $G_{3,3}$ content and an increase in the ratio of a- to b-series gangliosides were observed in extracts of rat brain (13) between embryonic days 18 and 20, but none of these changes could be ascribed definitively to neurons or to specific stages of neuronal development since whole brain tissue (which contains many types of neurons, all at different developmental stages) was analyzed. In hippocampal neurons, changes in ganglioside synthesis occur during axonogenesis and during the subsequent period of rapid axon growth. However, ganglioside synthesis does not appear to be a prerequisite for these events since inhibition of synthesis by either fumonisin B1 (34) or D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (35) has no effect on the formation of the initial axon between days 0 and 2 of culture (see Fig. 1 in Ref. 9). This suggests that the increase in complex ganglioside synthesis is a result of (rather than the cause of) axonogenesis. In contrast, inhibition of GSL synthesis does affect the formation of collateral axonal branches between days 2 and 3 of culture (9), during which time there is a shift toward the synthesis of a-series gangliosides.

Ganglioside Synthesis during Dendrite Growth—Surprisingly, between days 3 and 8 of culture, there is little alteration in the pattern of ganglioside synthesis, even though morphologically distinct dendrites form during this time. Although a minor ganglioside in hippocampal neurons, the synthesis of $G_{M2}$ appears somewhat elevated during this period (Table II), but the low levels of synthesis make reliable and accurate quantification difficult (see Fig. 4). If $G_{M2}$ is indeed elevated,
this would lend support to the idea, based on the accumulation of GM2 during ectopic dendrite formation in lysosomal storage diseases (36), that GM2 plays an important role in dendritogenesis. In cerebellar Purkinje cells, evidence has been provided, based on immunolocalization, that a minor ganglioside, GD1a, is specifically localized to proximal dendrites and cell bodies (37). Since we did not analyze the synthesis of this ganglioside in hippocampal neurons, we could not ascertain whether the synthesis of this or any other ganglioside that may be localized to dendrites is elevated during dendrite growth.

Ganglioside Synthesis during Synaptogenesis—Our recent observation that long-term inhibition of GSL synthesis does not effect the polarized segregation of axonal and dendritic markers in mature neurons (see Fig. 8 in Ref. 9) suggests that there is no direct correlation between the development of neuronal polarity and the synthesis of any major ganglioside. There is nevertheless a significant difference between the profile of gangliosides synthesized in immature neurons (i.e. days 0–3) and that observed in mature neurons with functional synapses (day 15). Whether gangliosides GM2, GD1a, GD1b, and GT1b play a particular role in synaptic function remains to be established.

In summary, we have shown that major changes in ganglioside metabolism and neuronal polarity occur during axonogenesis and axon elongation, but not during dendritogenesis and dendrite growth or synaptogenesis. Although our study does not address the function of gangliosides in these events, it does demonstrate that the rapid growth of axons results in an increase in complex and a-series gangliosides, whereas dendritogenesis and dendrite growth have little, if any, effect on ganglioside synthesis.

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