Restoration of Synthesis of Sulfoglucuronylglycolipids in Cerebellar Granule Neurons Promotes Dedifferentiation and Neurite Outgrowth*

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Sulfoglucuronyl carbohydrate (SGC) linked to the terminal moiety of neolacto-oligosaccharides is expressed in several glycoproteins of the immunoglobulin superfamily involved in neural cell-cell recognition as well as in two sulfoglucuronylglycolipids (SGGLs) of the nervous system. SGGLs and SGC-containing glycoproteins are temporally and spatially regulated during development of the nervous system. In the cerebellum, the expression of SGC, particularly that of SGGLs, is biphasic. Several studies have suggested that the initial rise and decline in the levels of SGGLs and SGC-containing proteins correlated with the migration of granule neurons from the external granule cell layer to the internal granule cell layer and their subsequent maturation, whereas the later rise and continued expression of SGGLs in the adult was associated with their localization in the Purkinje neurons and their dendrites in the molecular layer. Here it is shown by immunocytochemical methods that the expression of SGC declined progressively in granule neurons isolated from cerebella of increasing age. The decline in the expression of SGC in granule neurons was also shown with time in culture. These results correlated with the previously shown declining activity of the regulatory enzyme lactosylceramide N-acetylgalcosaminyltransferase (GlcNac-Tr) with age in vivo and in isolated granule neurons in culture. GlcNac-Tr synthesizes a key precursor, lactotriosylceramide, involved in the biosynthesis of SGGL-1. The downregulated synthesis of SGGLs in the mature granule neurons was shown by immunocytochemical and biochemical methods to be restored when a precursor, glucuronyleolactotetraosylceramide (GGL-1), which is beyond the GlcNac-Tr step, was exogenously provided to these cells. The biological effect of such restoration of the synthesis of SGGLs in the mature granule neurons leads to cell aggregation and enhanced proliferation of neurites, amounting to dedifferentiation.

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Sulfoglucuronyl carbohydrate (SGC)1 is expressed in several glycoproteins of the immunoglobulin superfamily, such as neural-cell adhesion molecules, L1, J1, cytotactin, TAG-1, myelin-associated glycoprotein, and P0, involved in neural cell recognition, and in two neolactoglycolipids (for reviews, see Refs. 1–3). A number of studies have suggested that SGC itself may be involved in cell recognition and signaling functions, especially during the development of the nervous system (4–10). We have characterized a specific SGC-binding protein, SBP-1 (sulfoglucuronyl carbohydrate-binding protein 1), from perinatal rat brain and have suggested that the biological function of SGC is mediated through SBP-1 (11, 12). Immunocytochemical and biochemical studies as well as confocal microscopy have shown that the expression of both SGC and SBP-1 is temporally and spatially coregulated in a stage-specific manner during perinatal development of the nervous system (11–17). In addition, topographically, these molecules are localized in apposing cellular structures of the perinatal rat cerebral cortex and cerebellum, suggesting a functional role in the processes of cell-cell interaction and cell migration (12, 17).

In the rat cerebral cortex, monoclonal antibody HNK-1-reactive sulfoglucuronylglycolipids (SGGLs) and SGC-containing glycoproteins are expressed strongly during embryonic stages

\[
\begin{align*}
\text{Glc}_{\beta 1}\text{-Cer} & \downarrow \text{Gal-Tr} \\
\text{Gal}_{\beta 1}-4\text{Glc}_{\beta 1}\text{-Cer} & (\text{LeOse}_{2}\text{Cer}) \downarrow \text{GlcNac-Tr} \\
\text{GlcNac}_{\beta 1}-3\text{Gal}_{\beta 1}-4\text{Glc}_{\beta 1}\text{-Cer} & (\text{LeOse}_{2}\text{Cer}) \downarrow \text{Gal-Tr} \\
\text{Gal}_{\beta 1}-4\text{GlcNac}_{\beta 1}-3\text{Gal}_{\beta 1}-4\text{Glc}_{\beta 1}\text{-Cer} & (\text{nLeOse}_{2}\text{Cer}) \downarrow \text{GlcA-Tr} \\
\text{GlcA}_{\beta 1}\text{-IV}_{3}\text{nLeOse}_{2}\text{Cer} & (\text{GGL-1}) \downarrow \text{SO}_{4}\text{-Tr} \\
\text{SO}_{3}\text{GlcA}_{\beta 1}\text{-IV}_{3}\text{nLeOse}_{2}\text{Cer} & (\text{SGGL-1})
\end{align*}
\]

**Scheme 1. Biosynthesis of SGGL-1.**

1 The abbreviations used are: SGC, sulfoglucuronyl carbohydrate; SGGL, sulfoglucuronylglycolipid; PD, postnatal day(s); EGCL, external granule cell layer; IGCL, internal granule cell layer; GlcNac-Tr, lactosylceramide N-acetylgalcosaminyltransferase; LeOse,Cer, lactotriosylceramide; GlcA-nLeOse,Cer, glucuronyleolactotetraosylceramide; FITC, fluorescein isothiocyanate; MAP, microtubule-associated protein; HPTLC, high-performance thin-layer chromatography; GFAP, glial fibrillary acidic protein.
SGGLs are maximally expressed around embryonic day 19 and almost completely disappear by postnatal day (PD) 10. In the cerebellum, SGGLs are expressed in a biphasic manner, with an initial peak at around PD1–3, followed by a second maximum at PD20 and continued expression in the adult (15). Biochemical (15) and immunocytochemical (16–18) analyses and studies with murine cerebellar mutants (19–21) with a characteristic loss of specific cell types have suggested that the initial decline in the levels of SGGLs correlated with the migration of immature granule neurons from the germinal zone in the external granule cell layer (EGCL) to the internal granule cell layer (IGCL), guided by Bergmann glial fibers. In contrast, the later rise and continued expression of SGGLs in the cerebellum was associated with their localization in the dendrites of the Purkinje cells in the molecular layer and their axon terminals in the deep cerebellar nuclei (17).

The in vivo expression of SGGLs, both in the cerebral cortex and in the cerebellum, is regulated by the enzyme lactosylceramide N-acetylgalactosaminyltransferase (GlcNAc-Tr), which synthesizes a key precursor, lactotriosylceramide (LcOse3Cer) (see Scheme 1) (22, 23). In the cerebral cortex, the activity of GlcNAc-Tr was down-regulated from a maximum at embryonic day 15 to an undetectable level by PD10, whereas the other enzymes involved in the subsequent steps for the synthesis of SGGLs were not as severely affected (1, 22–26). In the cerebellum, the activity of GlcNAc-Tr initially declined from birth to PD3, but subsequently increased up to PD15, explaining the observed initial decrease and then increase in the synthesis of SGGLs in the cerebellum.

In the cerebella of Purkinje cell-deficient adult mutant mice, in which Purkinje cells are lost, SGGLs and GlcNAc-Tr were absent, but the other three glycosyltransferases were not as affected (23). This indicated that in the normal adult cerebellum, GlcNAc-Tr and the latter three enzymes were localized in Purkinje cells, whereas only the latter three enzymes were localized in other cell types, such as in mature granule neurons of the IGCL and glial cells. The lack of SGGLs in the mutants was due to the absence of GlcNAc-Tr (23). Analyses of the enzymes in the specific microdissected cellular layers of normal adult rat also showed that the Purkinje cell layer and the molecular layer (where Purkinje cell dendrites are localized) contained all four enzymes (23). However, granule neurons and glial cells in the white matter lacked GlcNAc-Tr, but expressed the other three enzymes (23). It was concluded that the absence of SGGLs in adult granule neurons and glial cells was due to a specific deficiency of GlcNAc-Tr. Although adult granule neurons lacked GlcNAc-Tr and therefore SGGLs, isolated granule neurons from the perinatal cerebellum contained all four enzymes necessary for the synthesis of SGGLs (23). With development, the activity of GlcNAc-Tr in the isolated granule neurons declined, but the other enzymes were not as affected, indicating that immature granule neurons are capable of synthesizing SGGLs and that, with maturation, their synthesis is down-regulated (23).

In this study, we directly show that isolated immature granule neurons are able to synthesize SGC and that the expression of SGC is down-regulated with maturation of the cells in culture. In addition, we demonstrate that the down-regulated synthesis of SGGLs in the mature granule neurons is restored when a precursor such as glucuronylneolactotetraosylceramide (GlcA-nLcOse4Cer, GGL-1), which is beyond the GlcNAc-Tr step, is exogenously provided to these cells. The biological effect of such restoration of the synthesis of SGGLs in the mature granule neurons leads to dedifferentiation, cell aggregation, and enhanced proliferation of neurites.

**Synthesis of SGGLs in Granule Neurons**

**Materials**

Sprague-Dawley albino rats were purchased from Zivic-Miller Laboratories, Inc. (Pittsburgh, PA). Radioactive Na$_2$SO$_4$ (554 Ci/mol) was from NEN Life Science Products. Tissue culture media and substrates were from Life Technologies, Inc. All fine chemicals were purchased from Sigma or Fisher. Sulforafungurinylglycolipid SO$_4$-GlcA-nLcOse4Cer (SGGL-1) and glucuronylglucolipid GlcA-nLcOse4Cer (GGL-1) were prepared as described previously (27, 28). The hybridoma clone producing the HNK-1 IgM monoclonal antibody was from American Type Culture Collection (Rockville, MD). Anti-glial fibrillary acidic protein monoclonal antibodies and FITC-conjugated affinity-purified IgG secondary antibodies were from Boehringer Mannheim. Antibodies to microtubule-associated proteins (MAPs) raised in rabbit were from Sigma. Rhodamine-conjugated goat anti-mouse IgM secondary antibodies were from Cappel (Westchester, PA). The dilutions of the antibodies used were based upon the original hybridoma supernatant, serum from animals, or manufacturers’ supplied samples. Other chemicals and reagents have been reported in our previous articles (22–28).

**Purification of HNK-1 IgM Antibodies**

The supernatant of the HNK-1 hybridoma clone was mixed with an equal volume of EZ-SepA solution (Amersham Pharmacia Biotech) at pH 7.5 at room temperature. After 30 min, the precipitates formed were collected by centrifugation at 2000 × g for 30 min. The resulting pellet was resuspended in EZ-SepB solution, and the IgM was repurified with EZ-SepC solution. After 30 min, the precipitate was collected by centrifugation as partially purified IgM protein.

**Experimental Procedures**

**Preparation of HNK-1 Antisera**

Mixed cell cultures of neonatal rat cerebellum as well as enriched granule neuronal and astrocytic cell cultures were prepared essentially according to the methods of Hatten (29) as modified by Chou and Jungalwala (23). Briefly, dissociated cells from the cerebellum were separated on a three-step Percoll gradient (60, 35, and 20%) by centrifugation at 3300 rpm for 10 min in an IEC Centra-8R centrifuge. The granule cells were collected by centrifugation at 2000 × g for 30 min.

**Purification of HNK-1 IgM Antibodies**

The supernatant of the HNK-1 hybridoma clone was mixed with an equal volume of EZ-SepA solution (Amersham Pharmacia Biotech) at pH 7.5 at room temperature. After 30 min, the precipitates formed were collected by centrifugation at 2000 × g for 30 min. The resulting pellet was resuspended in EZ-SepB solution, and the IgM was repurified with EZ-SepC solution. After 30 min, the precipitate was collected by centrifugation as partially purified IgM protein.

**Dissociated Cerebellar Cell Cultures**

Mixed cell cultures of neonatal rat cerebellum as well as enriched granule neuronal and astrocytic cell cultures were prepared essentially according to the methods of Hatten (29) as modified by Chou and Jungalwala (23). Briefly, dissociated cells from the cerebellum were separated on a three-step Percoll gradient (60, 35, and 20%) by centrifugation at 3300 rpm for 10 min in an IEC Centra-8R centrifuge. The granule cells were collected by centrifugation at 2000 × g for 30 min.

**Granule Neuron-astrocytic Cell Population**

A mixed granule neuron-astrocytic cell population was isolated from the dissociated cells at the interface of the 20/60% Percoll gradient.

**Neuronal Cells under these conditions were killed, and the remaining cells were used as pure astroglial population. A mixed granule neuron-astrocytic cell population was isolated from the dissociated cells at the interface of the 20/60% Percoll gradient.**

**Biosynthesis of SGGL-1 in Granule Cells in Culture**

Granule cells isolated from PD5–7 rat cerebella were plated at a cell density of 1–2 × 10$^5$ cells/cover-slip for 4 h as described above. The coverslips were rinsed with Opti-MEM and transferred to 0.4 ml of medium (23) containing GGL-1 delivered to the cells via the lipid carrier α-cyclohextrin prepared as follows. Purified GGL-1 (4 μg) was dried from organic solvents, resuspended in 0.2 ml of ethanol, and mixed with α-cyclohextrin (1 mg/0.2 ml of ethanol/water (1:1)). The entire mixture was evaporated, and the residue was resuspended in 0.35 ml of culture medium (Opti-MEM) and sonicated in a sonicator bath for 1 min. Na$_2$SO$_4$ (125 nmol, 100 μCi) and horse serum (2.5% final concentration) were added. The cells were incubated at 37 °C overnight (or as stated otherwise) in an atmosphere of 95% air and 5% CO$_2$ in cell culture chambers. At the end of the incubation, the medium was removed, and the cells were washed once with Dulbecco’s buffered saline and detected by scraping.

The cells were then extracted with 6 ml of chloroform/methanol/H$_2$O (1:1:0.1). To the cell extracts, SGGL-1 (2 μg) was added as a carrier. The extracted samples were processed to isolate radioactive SGGL-1 as described previously (23). The isolated products were then analyzed by HPTLC with a developing solvent of chloroform, methanol, and 0.25% CaCl$_2$ (6:4:1) and autoradiography using Hyperfilm (Amersham Pharmacia Biotech) as described previously (25).
Immunocytochemistry

Cerebellar cells on coverslips at the appropriate stages, treated with and without GGL-1, were also analyzed by immunocytochemistry after fixing with 4% paraformaldehyde, pH 7.4, for 1 min at room temperature. Nonspecific binding was blocked with 5% normal goat serum for 10 min at room temperature. Cells were then exposed to the appropriate primary antibodies at room temperature for 15 min. Monoclonal antibody HNK-1 in the hybridoma supernatant, purified with EZ-Sep, was used at a 1:40 dilution. The cells were then exposed to rhodamine-conjugated goat anti-mouse IgM antibodies as described under “Experimental Procedures” and viewed through a fluorescence microscope. A–C, bright-field phase photomicrographs; D–F, fluorescent images of the same fields as in A–C, respectively. The fluorescent images in D–F were photographed under identical light exposure conditions.

Computer-assisted Image Analysis

Measurement of Fluorescence Intensity of Granule Cells—Coverslips of granule cells labeled with HNK-1 followed by fluorescent rhodamine-conjugated secondary antibodies were mounted on a Zeiss Axioplan fluorescence microscope with a 40× objective lens. Fields of cells were viewed and digitized according to optical density using a Hammamatsu Newvicon video camera and an IBAS image analysis system (Version 2.0, Zeiss/Kontron). Selected individual cells were traced, and an average optical density was determined for the total of all selected cells. Cell counts were also determined with the assistance of the computer pro-

Fig. 1. Expression of SGC antigens in granule neurons isolated from rat cerebella at different ages. Enriched populations of granule neurons were isolated as described under “Experimental Procedures” from PD3 (A and D), PD8 (B and E), and PD14 (C and F) rat cerebella. The cells were plated on polylysine-coated coverslips and allowed to adhere for 1 h. The cells were fixed and processed for immunocytochemistry with HNK-1 antibody followed by rhodamine-conjugated IgM secondary antibodies as described under “Experimental Procedures” and viewed through a fluorescence microscope. A–C, bright-field phase photomicrographs; D–F, fluorescent images of the same fields as in A–C, respectively. The fluorescent images in D–F were photographed under identical light exposure conditions.

Fig. 2. Expression of sulfoglucuronyl carbohydrate antigens in granule neurons isolated from PD5 rat cerebellum and grown in culture for 1 h (A and B) and 24 h (C and D). The cells were immunoreacted with HNK-1 and rhodamine-conjugated IgM antibodies as described for Fig. 1. A and C, bright-field phase photomicrographs; B and D, fluorescent images of the same fields as in A and C, respectively. The fluorescent images in B and D were photographed under identical light exposure conditions.

Prepared 0.1% p-phenylenediamine to prevent fading (30). The coverslips were viewed using a Zeiss Axioplan fluorescence microscope. In some experiments, sodium chlorate (5–30 mM) was added to the medium (to inhibit the synthesis of SGGL-1 from added GGL-1) to evaluate the effect of added GGL-1 by itself on granule neuron cell morphology and aggregation.
gram. The average background pixel optical density was determined for regions of each image not containing cells to determine background for substraction. For each culture coverslip, 15–30 cells from three to five different randomly selected fields were analyzed, and the data were averaged. For each paradigm measured, at least two separate experiments were performed.

**Measurement of Neurite Length of Granule Cells**—Measurement of neurite length of granule cells was done with coverslips of granule cell cultures labeled with anti-MAP antibodies followed by FITC-conjugated secondary antibodies. Images were digitized according to optical density as described previously. All visible neurites were traced initially by an automated segmentation algorithm and edited manually for accuracy. The number of granule cells in the field was also determined. The sum of all traced neurites and a cell count for each field were determined. For each cell culture coverslip, three randomly selected fields were analyzed, and the data were averaged.

**RESULTS**

**Expression of SGC Declines in Isolated Granule Cells during Development**—SGC-containing glycoproteins and glycolipids are recognized by monoclonal antibody HNK-1 (27). The expression of SGC in isolated cerebellar granule cells from postnatal day 3, 8, and 14 rats was studied by plating the cells in culture for 1 h. The cells were then exposed to HNK-1 antibody followed by rhodamine-conjugated IgM secondary antibodies and analyzed by fluorescence microscopy (Fig. 1). Practically all granule neurons as visualized by bright-field phase microscopy were HNK-1-reactive. The reactivity of HNK-1 was highest with granule cells isolated from cerebella of PD3 animals (Fig. 1D). This reactivity declined in the cells from PD8 animals (Fig. 1E) and almost completely disappeared in the cells isolated from PD14 rats (Fig. 1F). The results show that the expression of SGC was down-regulated with the animal age in granule cells.

**Expression of SGC Declines in Isolated Cells with Time in Culture**—Purified granule cells isolated from PD5 rat cerebellum were maintained in culture from 1 to 24 h, and the expression of SGC in the cells was monitored with HNK-1 antibody. The significant expression of SGC in granule neurons after 1 h in culture (Fig. 2B) declined severely after 24 h in culture (Fig. 2D).

**Expression of SGC in Isolated Astroglial Cells from Rat Cerebellum**—Astroglial cells were isolated from PD4 rat cerebellum and purified by panning on polylysine-coated coverslips. The isolated cells after 24 h in culture were double-stained with HNK-1 and anti-GFAP antibodies followed by rhodamine- and FITC-conjugated secondary antibodies, respectively. SGC was expressed strongly only in a small population of astroglial cells (Fig. 3B) since several GFAP-positive cells (Fig. 3C) did not express HNK-1-reactive SGC.

**Restoration of SGC Synthesis in Granule Cells by Exogenously Adding GGL-1**—A mixed population of granule neurons and astroglial cells from PD4 rats was maintained in culture for 16 h. The cultures were then exposed to 4 µg of GGL-1 in the medium along with the lipid carrier α-cyclohextrin for 10 h. The control cultures were exposed to α-cyclohextrin in the medium without GGL-1. Both the control (unexposed) and GGL-1-exposed cells were treated with HNK-1 antibody. SGC was not expressed in granule neurons of the control cultures, except some astroglial cells expressed SGC (Fig. 4B). However, granule neurons of cultures exposed to GGL-1 strongly expressed SGC and showed extensive neurite outgrowth (Fig. 4, C and D). The SGGL-expressing cells also appeared to aggregate more readily (Fig. 4, C and D). There was no effect when other glycolipids, such as glucosylceramide, lactosylceramide, LcOse<sub>3</sub>Cer, globoside, and paragloboside, were tested instead of GGL-1.

The relative intensity of the rhodamine fluorescence due to reactivity of SGC with HNK-1 was measured with a digital camera and a computer-assisted image analysis system. The relative fluorescence intensity due to SGC in granule cells increased with the amount of GGL-1 added to the medium (Fig. 5A). The fluorescence intensity due to SGC in granule neurons was also dependent upon the amount of α-cyclohextrin added along with a fixed amount of GGL-1 (data not shown). 1 mg of α-cyclohextrin in 0.4 ml of medium was optimal. Higher levels of cyclohextrin were damaging to the cells. The relative fluo-
Synthesis of SGGLs in Granule Neurons

Fig. 4. Restoration of expression of sulfogluconeryl carbohydrate in granule neurons by exogenous addition of precursor glucononylneolactotetraosylceramide (GGL-1). Mixed populations of granule neurons and astroglial cells from PD4 cerebellum were grown in culture for 16 h. Experimental cultures were provided with GGL-1 (4 μg) along with the carrier cyclodextrin in the culture medium, whereas control cultures received only cyclodextrin. The cultures were maintained for an additional 10 h and immunoreacted with HNK-1 and rhodamine-conjugated IgM secondary antibodies as described under “Experimental Procedures.” A and B, control cultures; C and D, experimental cultures with added GGL-1. A and C, phase light photomicrographs; B and D, fluorescent images of the same fields as in A and C, respectively.

Fig. 5. A, increase in the level of sulfogluconeryl carbohydrate expression in granule neurons with increasing amounts of added GGL-1. The culture conditions of the experiment were the same as described for Fig. 4, except the amount of added GGL-1 was varied as shown. The cultures were immunoreacted with HNK-1 followed by rhodamine-conjugated IgM secondary antibodies under identical conditions. The relative fluorescence intensities of the granule cells due to SGG1–1 conjugated IgM secondary antibodies under identical conditions. The culture conditions of the experiment were the same as described for “Experimental Procedures.” After 48 h of incubation, the radioactive SGGL-1 formed was analyzed by HPTLC followed by autoradiography. Lanes 1–8, autoradiogram of the HPTLC; lanes 9–11, HPTLC of standards sprayed with orcinol. Lane 1, mixed population of granule neurons and astroglial cells incubated without cycloexdrin and GGL-1; lane 2, same cells as in lane 1 with cycloexdrin, but without GGL-1; lane 3, same cells as in lane 1 with GGL-1, but without cycloexdrin; lane 4, same cells as in lane 1 with GGL-1 and cycloexdrin; lane 5, enriched population of astroglial cells with GGL-1 and cycloexdrin; lane 6, purified population of granule neurons with GGL-1 and cycloexdrin; lane 7, same protocol as in lane 4, but in a different experiment; lane 8, pure population of astrocytes grown in culture for 2 weeks with GGL-1 and cycloexdrin; lane 9, sulfatide standard; lane 10, SGGL-1 standard; lane 11, GGL-1 standard. Experiments pertinent to lanes 1–6 and lanes 7 and 8 were performed at different times; therefore the migrations of SGGL-1 bands in all lanes are not identical. Doublets seen for standards SGGL-1 and GGL-1 (lanes 10 and 11) are due to the presence of small amounts of SGGL-2 and GGL-2, respectively.

Biochemical Characterization of the Product Synthesized

from GGL-1 by Granule Neurons and Astroglial Cells—The biosynthesis of SGGL-1 by different cerebellar cell types from precursor GGL-1 and Na235SO4 in the medium was studied by HPTLC and autoradiography. The mixed granule neuron-astroglial cell population of cells synthesized radioactive SGGL-1 from GGL-1 and Na235SO4 in the medium (Fig. 6, lanes 4 and 7). However, these cells failed to synthesize SGGL-1 in the absence of cycloexdrin and/or GGL-1 (Fig. 6, lanes 1–3). These results show that cycloexdrin was required for the transport of GGL-1 across the plasma membrane of the cells. Enriched astroglial cells and granule neurons as well as pure populations of astrocytes grown in culture for 2 weeks also synthesized SGGL-1 (Fig. 6, lanes 5, 6, and 8, respectively).

The mixed cell population as well as the enriched astroglial fraction also synthesized sulfatide, presumably from endogenous cerebroside and added Na235SO4 (Fig. 6, lanes 1–5 and 7).
The biosynthesis of SGGL-1 in mixed populations of granule neurons and astroglial cells in culture supplied with increasing amounts of GGL-1 in the medium. The experimental conditions were the same as described for Fig. 6, except that increasing amounts of GGL-1 were added as shown. The radioactive SGGL-1 synthesized was analyzed by HPTLC and autoradiography (see inset) followed by computer-assisted optical density measurements of the SGGL-1 bands in the autoradiogram as described previously (23). In the inset, lanes 1–5 correspond to 0, 1.25, 2.5, 4.0, and 5.0 μg of GGL-1 added to the medium, respectively.

**FIG. 7.** Biosynthesis of SGGL-1 in mixed populations of granule neurons and astroglial cells in culture supplied with increasing amounts of GGL-1 in the medium. The experimental conditions were the same as described for Fig. 6, except that increasing amounts of GGL-1 were added as shown. The radioactive SGGL-1 synthesized was analyzed by HPTLC and autoradiography (see inset) followed by computer-assisted optical density measurements of the SGGL-1 bands in the autoradiogram as described previously (23). In the inset, lanes 1–5 correspond to 0, 1.25, 2.5, 4.0, and 5.0 μg of GGL-1 added to the medium, respectively.

**FIG. 8.** Computer-assisted image analysis of neurite length of granule cells in culture in the absence and presence of added GGL-1 in the medium.

<table>
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<tr>
<th>Coverslip</th>
<th>Total neurite length</th>
<th>No. of granule neurons analyzed</th>
<th>Neurite length/μ cell</th>
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<tr>
<td>Control</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>166±9</td>
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<td>414±5</td>
<td>35</td>
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<td>Mean</td>
<td>207±48</td>
<td>28±4</td>
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GGL-1 treated

<table>
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<th>No. of granule neurons analyzed</th>
<th>Neurite length/μ cell</th>
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<tr>
<td>Mean</td>
<td>394±73</td>
<td>57±5</td>
<td>16.0±1.0*</td>
</tr>
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*Granule neurons in culture on coverslips were immunostained with anti-MAP antibodies followed by FITC-conjugated secondary antibodies. The cultures were viewed on the computer screen with the aid of a fluorescence microscope, video camera, and the IBAS computer-assisted image analysis system. The fluorescent neurites were traced, and the total neurite length as well as the number of granule neurons were determined. For each culture coverslip, three different randomly selected fields were analyzed, and the averages are presented here. Each coverslip represents a separate experiment.

The mean ± S.E. of neurite length per granule neuron of control cultures was significantly different from that of GGL-1-treated cultures by analysis of variance t test, with p values of <0.02.

However, the synthesis of sulfatide in granule neurons and pure astrocytes was minimal (Fig. 6, lanes 6 and 8, respectively), indicating minimal contamination of these cells by oligodendroglial cells, which specifically synthesize sulfatide.

The synthesis of radioactive SGGL-1 by the mixed cell population was dependent upon the amount of GGL-1 added to the medium (Fig. 7). The synthesis of radioactive sulfatide was affected minimally by addition of GGL-1 between 2.5 and 5.0 μg (Fig. 7, inset).

**Effect of Restoration of Synthesis of SGGL-1 in Granule Neurons on Cell Morphology**—The morphological effect of the restoration of synthesis of SGGL-1 in granule neurons by addition of GGL-1 was measured. After addition of 4 μg of GGL-1 and cell culture for 16 h, the control and experimental cells were processed with antibodies to MAPs followed by FITC-conjugated secondary antibodies to identify granule neurons and their cell processes (neurites). The lengths of the neurites were determined by computer-assisted image analysis, and the total length of neurites in all the cells and the average length of the neurite per granule cell were calculated (Fig. 8 and Table I). The mean total neurite length for about the same number of granule neurons treated with GGL-1 was about twice that of untreated cultures. The average neurite length per granule neuron was more than double for the GGL-1-treated culture compared with the untreated cultures.

To evaluate if the granule neuron cell morphology was altered due to synthesis of SGGL-1 or due merely to added GGL-1, the synthesis of SGGL-1 from GGL-1 was suppressed by inhibiting the activity of the enzyme GGL-1 sulfotransferase by sodium chlorate. Previously, it has been reported that up to 30 mM chlorate ions specifically inhibited the synthesis of 3′-phosphoadenosine phosphosulfate, the active donor of sulfate, without significantly affecting growth and viability of cells in culture (31, 32). Granule neurons from PD5 rat cerebellum, when treated with 0–30 mM chlorate in the medium, did not appear to have significant toxic effect up to 48 h. Granule...
neurons treated with 20 mM chlorate failed to synthesize SGGL-1 from added GGL-1 (Fig. 9E); however, without chlorate, they synthesized a significant amount of SGGL-1 as visualized by immunocytochemistry with HNK-1 antibody (Fig. 9C). Neurite outgrowth and cell aggregation, as visualized with anti-MAP antibodies, were significantly increased in cells treated with GGL-1 (Fig. 9D) compared with cells without GGL-1 (Fig. 9B). However, in the presence of chlorate and GGL-1, neurite outgrowth and cell aggregation were almost similar (Fig. 9F) to cells without added GGL-1 (Fig. 9B). The results show that the synthesis of SGGL-1 from GGL-1 was required for the alteration of morphological characteristics and cell aggregation and that GGL-1 by itself did not have similar effects.

DISCUSSION

Adult mammalian cerebellum is composed of four major cell types. Granule neurons are >80% of the total neuronal population, and they form the major portion of the IGCL. Purkinje neurons are ~10% of the total neuronal population and are localized at the junction of the IGCL and molecular layer (33). The dendrites of the Purkinje neurons form the molecular layer. Oligodendroglia and astroglia are the two glial cell types. Oligodendroglia generate myelin membranes and are mostly concentrated in the white matter layer. Astroglia are primarily located in the IGCL; however, a specific type of astroglia, viz. Bergmann glia, and their processes span the molecular layer into the EGCL especially during neonatal development.

Neuroanatomical studies (33) have shown that during early development of the rodent cerebellum (embryonic day 17 to PD3), granule neurons start to proliferate over the entire surface of the primordial cerebellum in the EGCL. The EGCL reaches its peak in size by PD6–7. During this period, a portion of the granule neurons start migrating internally from the EGCL, along the Bergmann glial fibers, to reside in the IGCL. By PD21, almost all granule neurons complete their migration, leaving the EGCL to regress and disappear. This cellular migration is cued by cellular interactions; however, the precise nature of the molecules involved is mostly unknown. The characterization of such molecules is important not only in defining normal cerebellar development, but also in understanding steps that cause arrested or abnormal migration as manifested in several neuronal migration disorders, leading to severe mental retardation and profound developmental disabilities in humans.

SGC has been implicated previously in the migration of neural crest cells (4) as well as in the upward migration of neuronal cells from the proliferating ventricular zone to their final destination in the cortical plate in the cerebral cortex (11, 12). The expression of SGC is down-regulated after cell migration is completed. Our biochemical and immunocytochemical studies have shown that, analogous to that in the cerebral cortex, the expression of SGC declines in the IGCL of the cerebellum after the granule neuron migration and maturation are completed.

Previously, we have shown that the down-regulation of SGC, particularly that of SGGLs, with development in vivo both in the cortex and in the cerebellum is due to declining activity of the enzyme GlcNAc-Tr (22). Similarly, the GlcNAc-Tr activity was down-regulated in granule neurons isolated from PD3–14 animals (23). The results presented in Fig. 1 show that a significant level of SGC present in the isolated granule neurons from PD3 animals declined in the cells from PD8 animals and was almost completely absent in the granule neurons from PD14 animals. Thus, immature granule neurons appeared to have SGC, which was down-regulated with maturation. Some, but not all, isolated astroglial cells also show SGC expression (Fig. 3), indicating that different astrocytes may be at dissimilar stages of maturation in the isolated population.
Previous studies in vivo and in vitro have indicated that although mature granule cells lack GlcNAc-Tr, and therefore SGGLs, they do have significant activities of the enzymes involved in the subsequent steps of the biosynthesis of SGGLs, as shown in Scheme 1 (22–26).

Here we have shown that when isolated intact mature granule cells were provided with precursor GGL-1, they were able to synthesize SGGL-1 (Figs. 4–7). Precursors such as LcOse,Cer or nLcOse,Cer beyond the GlcNAc-Tr step were also added in the medium similar to GGL-1, and the cells were analyzed by immunocytochemistry with anti-nLcOse4Cer (1B2) and HNK-1 antibodies as well as by biochemical analysis. For the latter, the cells were extracted and analyzed for the synthesis of nLcOse,Cer from LcOse,Cer by HPTLC and immunoblotting with 1B2 antibodies and by [14C]galactose incorporation as well as for the synthesis of SGGL-1 from both these precursors by 35SO4 incorporation. We were unable, however, to show the expected synthesis of nLcOse,Cer or SGGL-1 under the conditions of the assays. It is likely that these precursors were not taken up by the live cells or, if taken up, did not reach the correct site in the endoplasmic reticulum and Golgi apparatus where the synthesis is known to occur. It is also likely that competing glycosyltransferases, such as β1–3-galactosyltransferase, sialyltransferase, or fucosyltransferase, that act on LcOse,Cer and nLcOse,Cer to synthesize other glycolipids would diminish the level of the final product (SGGL-1) below the limits of detection. In addition to granule neurons, the pure population of astroglial cells also synthesized SGGL-1 when supplied with exogenous GGL-1 in the medium, indicating that these cells also carry the specific GGL-1 sulfotransferase required for the synthesis of SGGL-1.

The restoration of the synthesis of SGGL-1 in the mature granule cells on addition of GGL-1 had remarkable morphological consequences. Restoration had no toxic effects on the viability of the cells. Granule neurons restored with the synthesis of SGGL-1 had more than double neurite outgrowth compared with unrestored neurons. In addition, the former appeared to aggregate more than the latter. The results suggest that SGGL-1 had either directly or indirectly altered the differentiation stage of the granule neurons. When other lipids were added to the medium, no change in cell morphology was observed, suggesting the requirement of the specificity of SGGL-1. GGL-1 by itself appeared to have little effect on neurite outgrowth and cell aggregation when added to the medium in the presence of chlorate to prevent its conversion to SGGL-1. This is perhaps the first report demonstrating that the induction of the synthesis of a membrane lipid can alter the morphology and differentiation stage of primary neuronal cells in cultures. It would be of interest to determine the effect of such manipulation on cell morphology and migration in vivo.

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REFERENCES

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