Association of Src Family Tyrosine Kinase Lyn with Ganglioside GD3 in Rat Brain

POSSIBLE REGULATION OF Lyn BY GLYCOSPHINGOLIPID IN CAVEOLAE-LIKE DOMAINS*

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Association of gangliosides with specific proteins in the central nervous system was examined by co-immunoprecipitation with anti-ganglioside antibody. Protein kinase activity was detected in precipitates with monoclonal antibody to ganglioside GD3 (R24) from membrane fraction of rat brain. Using in vitro kinase assay, several phosphorylated proteins of 40, 53, 56, and 80 kDa were isolated by gel electrophoresis. Of these proteins, the proteins of 53 and 56 kDa (p53/56) were identified as two isoforms of Src family tyrosine kinase Lyn, based on co-migration during gel electrophoresis, comparative peptide mapping, and sequential immunoprecipitation with anti-Lyn antibody. The identification was confirmed using a cDNA expression system in Chinese hamster ovary (CHO) cells, which express solely ganglioside GM3, the enzymatic substrate of GD3 synthase. In cotransfection with GD3 synthase and Lyn expression plasmids, R24 immunoprecipitated Lyn and anti-Lyn antibody immunoprecipitated GD3. R24 treatment of rat primary cerebellar cultures induced Lyn activation and rapid tyrosine phosphorylation of several substrates including mitogen-activated protein kinases. Furthermore, sucrose density gradient analysis showed that Lyn of cerebellum and CHO transfectants were detected in a low density light-scattering band, i.e. the caveola membrane fraction. R24 immunoprecipitated caveolin from Triton X-100 extract of CHO transfectants. These observations suggest that GD3 may regulate Lyn in a caveolae-like domain on brain cell membranes.

Gangliosides, sialic acid-containing glycosphingolipids, are found in the outer leaflet of the plasma membrane of all vertebrate cells and are thought to play functional roles in cellular interactions and control of cell proliferation (1–4).

In the nervous system, where gangliosides are especially enriched, the species and amounts of gangliosides undergo profound changes during development, suggesting that they may play fundamental roles in this process (5). The accumulation of gangliosides within the neurons in ganglioside storage disease results in extensive neurite growth (6). Exogenously administered gangliosides have been shown to accelerate regeneration of the central nervous system in vivo after lesioning (7). The addition of exogenous gangliosides or anti-ganglioside antibody to the primary neurons and neuroblastomas in vitro has been shown to stimulate the differentiation with concomitant neurite sprouting and extension (8–10). Transfection of ganglioside GD3 (NeuAco2,8NeuAco2,3Galβ1,4Glcβ1,1-ceramide)1 synthase cDNA into neuroblastoma induced cholinergic differentiation with neurite sprouting (11). The differentiation caused by anti-ganglioside antibody is due to increased cAMP accumulation and activation of protein kinase A (10). These data suggest that ganglioside could modulate a signaling pathway of neuronal differentiation. However, molecular mechanisms of the ganglioside-dependent neuronal differentiation remain obscure.

We have been investigating a biosynthesis of gangliosides during normal neuronal development and oncogenic transformation (12–20). Ganglioside biosynthesis takes place in the Golgi apparatus, where glucosylceramide is glycosylated by sequential addition of galactose, sialic acid, and N-acetylgalactosamine. Ganglioside GD3 is important as a precursor of the b and c series ganglioside. Recently we isolated GD3 synthase (α2,8-sialyltransferase) cDNA and found that the GD3 synthase expression was regulated in stage- and spatio-restricted manners in the rat central nervous system (17, 19). GD3 is the predominant ganglioside of the early, immature nervous system of birds and mammals, but its amount decreases in contrast with the accumulation of higher sialylated gangliosides during maturation (21). GD3 is implicated in cell attachment (22) and cell-to-cell interactions during embryogenesis (23). Non-receptor protein-tyrosine kinases of the Src subfamily are implicated in signal transduction systems that control cell proliferation and differentiation (24). In the present study, we isolated ganglioside GD3 binding proteins from rat brain to clarify the ganglioside-mediated signal transduction and identified two of them as Src family tyrosine kinase Lyn.

EXPERIMENTAL PROCEDURES

Materials—The mouse IgG3 anti-ganglioside GD3 monoclonal antibody R24 was obtained from hybridoma R24 (American Type Culture Collection no. HB8445). Anti-Lyn (Lyn8), anti-Fyn (Fyn301), and anti-Yes (3H9) monoclonal antibodies were purchased from Wako Chemicals (Osaka, Japan). Anti-Src monoclonal antibody (GD11) was a gift from Dr. Y. Fukui (Faculty of Life Science and Agriculture, University of Tokyo). Anti-Lyn rabbit polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-caveolin polyclonal antibody and horseradish peroxidase-conjugated anti-phosphotyrosine antibody (PY20) were purchased from Transduction Laboratories (Lexington, KY).

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KY). Fluorescein isothiocyanate (FITC)\textsuperscript{2}-conjugated goat anti-mouse IgG antibody was purchased from Zymed (San Francisco, CA). Phosphospecific mitogen-activated protein kinase (MAPK) antibody was purchased from New England BioLabs (Beverly, MA). Triton X-100 and EGTA were purchased from Sigma.

**Primary Culture**—Cultures were prepared from cerebellum of 7-day-old rats as described by Yuzaki (25). In brief, cells were isolated by trypsinization, followed by trituration in DNase solution; they were then suspended in Fischer’s serum-free medium.

**Immunoprecipitation and In Vitro Kinase Assay**—Membrane fraction was prepared from adult Wistar rat brain. Brains were homogenized in ice-cold buffer A (0.32 M sucrose 1 mM Tris-HCl, pH 7.4, 0.1 mM EDTA) using a Teflon motor-driven glass homogenizer. The homogenate was centrifugated at 900 \( g \) for 10 min. The supernatant was centrifugated at 11,500 \( g \) for 20 min. The postnuclear supernatants were collected at 14,000 rpm for 3 min. Aliquots (0.5 ml, 750 \( \mu \text{g of protein} \) of the supernatants were precleared with protein G-Sepharose (7.5 \( \mu l \)), and then incubated with anti-G D3 antibody R24 (2.5 \( \mu l \)) for 1 h and precipitated with protein G-Sepharose (7.5 \( \mu l \)). Following immunoprecipitation, the beads were washed three times with wash buffer, washed once with kinase buffer (30 mM HEPES, pH 7.5, 10 mM MgCl\(_2\), 2 mM MnCl\(_2\)), and resuspended in 20 \( \mu l \) of kinase buffer. The reaction was started by addition of 5 \( \mu l \) of [\( \gamma \text{-}^{32}\text{P} \)]ATP (3,000 Ci/mmol, NEN Life Science Products) and incubated for 10 min at room temperature. Phosphorylation was stopped by the addition of Laemmli sample buffer, and samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by autoradiography. In a re-immunoprecipitation experiment, after the kinase reaction the samples were boiled for 5 min in lysis buffer with 1% SDS, diluted 10-fold with lysis buffer, and then re-immunoprecipitated with anti-Src family kinase antibodies or R24.

**Phosphoamino Acid Analysis**—Phosphoamino acid analysis was performed as described (26). Phosphoprotein radiolabeled with \( ^{32}\text{P} \) (p53/56) was eluted from a polyacrylamide gel and hydrolyzed in 6 \( M \) hydrochloric acid at 105 \( ^\circ \text{C} \) for 2 h. The hydrolysate was evaporated and resuspended in 50 \( \mu l \) of carrier phosphoamino acid solution containing 1 \( M \) phosphotyrosine, phosphothreonine, and phosphoserine. The solution was then subjected to cellulose thin layer chromatography (TLC) with a developing solution consisting of 1-butanol, isopropanol, acetic acid, and water (3:1:1:1). The plate was dried, sprayed with ninhydrin to determine the positions of phosphoamino acids, and then subjected to autoradiography.

**Flow Cytometry**—Rat primary cerebellar cultures were harvested 24 h after seeding by pipetting. The cells were treated first with R24 and then with FITC-conjugated anti-mouse IgG antibody and were analyzed on a FACScan (Becton-Dickinson).

**Expression of Src Family Tyrosine Kinases in CHO Cells**—Chinese hamster ovary (CHO) cells were transfected transiently (48 h) with a pME18S expression plasmid (27) containing Lyn (28) or c-Src (29) cDNA using LipofectAMINE™ (Life Technologies, Inc.) according to the manufacturer’s instructions. Expression of transgenes was confirmed by immunoblotting. Lyn was not detected endogenously in CHO cells. Although Src was endogenously detected in CHO cells, transient expression greatly enhanced Src. CHO cells express solely the ganglioside G\(_{\text{D3}}\), an enzymatic substrate of G\(_{\text{M}}\) synthsase. We previously established a CHO cell line, CST, expressing G\(_{\text{D3}}\) synthase and synthesizing G\(_{\text{D3}}\) constitutively by stable transfection of full-length human G\(_{\text{M}}\) synthase cDNA (20). In this experiment, we used CST cells as G\(_{\text{D3}}\)-positive cells and parental CHO cells (CHO expressing polyoma large T) as G\(_{\text{D3}}\)-negative cells (17).

**Labeling of Cellular Glycosphingolipids, Lipid Extraction, and TLC**—Metabolic labeling of glycosphingolipids was performed using 2 \( \mu\text{Ci/ml} \) [[\( ^{3} \text{H} \)]galactose (300 \( \mu\text{Ci/mmole} \), NEN Life Science Products) for 24 h. Lipids in immunoprecipitates were extracted with chloroform/methanol (1/1, v/v) by sonication and separated on a silica gel TLC in a solvent system of chloroform, methanol, and 0.5% CaCl\(_2\). 1M M phosphotyrosine (Fig. 2).

**RESULTS**

**Detection of Protein Kinase Activity in Immunoprecipitates with Anti-
G\(_{\text{D3}}\) Antibody**—Immunoprecipitates with anti-G\(_{\text{D3}}\) antibody (R24) from Triton X-100 extract of adult rat brain membrane were analyzed for the presence of protein kinase activity by an in vitro kinase assay. In vitro kinase reaction resulted in phosphorylation of several proteins of 40, 53, 56, and 80 kDa, as judged by SDS-PAGE (Fig. 1). No kinase activity was detected in R24 precipitates performed in the presence of 30 \( \mu\text{M} \) G\(_{\text{M}}\) or in immunoprecipitates with control mouse IgG. The present study dealt with identification of 53- and 56-kDa proteins (p53/56). The \( ^{32}\text{P} \)-labeled p53/56 were eluted from SDS-PAGE and hydrolyzed with 6 \( M \) hydrochloric acid. The hydrolysate was separated by thin layer chromatography. Radioactivity was detected only in the position of phosphotyrosine (Fig. 2).

**Identification of p53/56 as the Src Family Tyrosine Kinase Lyn**—The molecular weight and tyrosine phosphorylation of p53/56 suggested that it could be a Src family tyrosine kinase. To investigate this possibility, we have compared the SDS-PAGE patterns of in vitro phosphorylated proteins that were
immunoprecipitated from membrane fraction of rat brain by antibodies specific to several members of the Src tyrosine kinase family, i.e. Src, Fyn, Yes, and Lyn, to that of G_{D3} specific antibody R24. The pattern of phosphorylated proteins obtained with anti-Lyn antibody resembled most closely the protein pattern resulted from R24 immunoprecipitation, with p53/56 migrating exactly as two autophosphorylated splice isoforms, p53^{40kDa} and p56^{80kDa}, of Lyn (Fig. 3A). Phosphorylation of 40- and 80-kDa proteins were also observed in immunoprecipitates by anti-Lyn antibody.

The bands of p53/56 and p53/56^{40kDa} were excised and subjected to comparative peptide mapping after digestion with V8 protease. As shown in Fig. 3B, the maps of p53/56 and p53/56^{40kDa} appeared indistinguishable. The maps differed from those of Src, Fyn, and Yes (data not shown).

The identification was confirmed by sequential immunoprecipitation with R24 and anti-Lyn antibody. The in vitro kinase assay was performed with R24 immunoprecipitates, after which the immune complexes were disrupted by boiling in SDS-containing buffer and subjected to a second immunoprecipitation with antibodies specific to Src, Fyn, Yes, and Lyn. Anti-Lyn antibody, but not anti-Src, Fyn, or Yes antibodies, precipitated specifically the p53/56 in re-immunoprecipitation experiments (Fig. 3C). R24 did not precipitate p53/56 after SDS boiling, indicating that R24 does not bind p53/56 directly.

**Association of Lyn with G_{D3} in a cDNA Expression System—**
The association of Lyn with G_{D3} was confirmed using a cDNA expression system in CHO cells. G_{D3} (NeuAc2,3Galβ1, 4Glcβ1,1-ceramide) is the only ganglioside synthesized in CHO cells and is an enzymatic substrate of G_{D3} synthase. We have previously established an CHO cell line, CST, constitutively expressing G_{D3} synthase (20). Both CHO and CST cells were transfected transiently with an expression plasmid carrying cDNA for a p56 splice isoform of Lyn. Lyn activity was co-precipitated by R24 from CST cells that constitutively synthesized G_{D3}, but not from CHO cells (Fig. 4A). In a control transfection with a Src expression plasmid, R24 did not precipitate Src activity in CST cells expressing Src (Fig. 4B). This finding suggests that R24 does not precipitate the whole membrane protein.

To substantiate further the association of Lyn with G_{D3}, CST cells overexpressing p56^{40kDa} were metabolically labeled with [1^{14}C]galactose. After immunoprecipitation with anti-Lyn antibody, lipids were extracted from the co-immunoprecipitates and subjected to TLC and autoradiography. [1^{14}C]-Labeled G_{D3} was detected in the immunoprecipitate with anti-Lyn antibody, but not with control mouse IgG. Therefore, we conclude that ganglioside G_{D3} associates with Src family tyrosine kinase Lyn in rat brain.

**Association of Lyn with G_{D3} in Primary Cerebellar Cultures—** Lyn is known to be localized in cerebellar granule cells

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we measured Lyn activity in primary cerebellar cultures after treatment with R24. For this, Triton X-100 extracts were prepared from primary cerebellar cultures, which were treated with R24 for 0.5–30 min. Immunoprecipitation was carried out with anti-Lyn antibody. Kinase activity was measured by \textit{in vitro} autophosphorylation. R24 treatment resulted in a rapid (within 1 min) and significant (3-fold) increase of Lyn activity, with no change in the amount of Lyn protein (Fig. 6).

**R24 Induces Protein-tyrosine Phosphorylation in Primary Cerebellar Cultures**—A possible increase in tyrosine phosphorylation of cellular proteins as a result of treatment of rat primary cerebellar cultures with R24 was investigated. After incubation with R24, cells were extracted with 1% Triton X-100 and cell extracts were fractionated into supernatant and particulate fraction. After SDS-PAGE, phosphorysotines were detected by anti-phosphotyrosine immunoblotting. Treatment with R24 induced tyrosine phosphorylation of several proteins in the supernatants, including a prominent phosphorylation of a protein of about 80 kDa (Fig. 7). The phosphorylation peaked at 1 min and returned to the control level at 30 min. One of the detected proteins was identified as MAPK, using phosphospecific MAPK antibody. The antibody detects p42 and p44 MAPK only when it is catalytically activated by phosphorylation at Tyr-204. The phosphorylation peaked at 5 min and returned to the control level at 30 min. This observation suggests that R24 stimulates the MAPK cascade via a Lyn signaling pathway.

**Sucrose Density Gradient Analysis**—Ganglioside is known to form clusters in the outer leaflet of the lipid bilayer (32). Lyn anchors on the inner leaflet via N-terminal lipid modification, palmitoylation, and myristoylation (33). How does \(G_{D3}\) associate with Lyn? The association is probably due to the presence of detergent-insoluble glycosphingolipid-enriched complex (DIG) or caveolae-like domains on the cell surface of the brain (34). These complexes are known to be enriched in Src family tyrosine kinase, and can be isolated as a low density light-scattering band by sucrose density gradient analysis. Therefore, we investigated the distribution of Lyn in a sucrose density gradient. Subcellular fractionation of extracts from either rat cerebellum or CST cells transfected with \(p56^{\text{Lyn}}\) was performed in parallel. Most of Lyn from a rat cerebellum and about 50% of Lyn from CST cells in a 150-mm dish was present in the light scattering band at fractions 3–5 (Fig. 8, C and F, respectively). This discrepancy probably derives from differences in the protein/detergent ratio during the initial homogenization, as has recently been pointed out (35), because most of Lyn and about 70% of Src from the transfectants in 10 150-mm dishes were present in the light scattering band (data not shown). In the CST cells, caveolin (a marker protein of caveolae) was also present in the low density fraction (Fig. 8G). Caveolin was not detected in homogenate of cerebellum (data not shown).

**Immunoprecipitation of Caveolin with R24**—We investigated whether R24 immunoprecipitated caveolae of CHO cells. In CHO cells transfected with \(G_{D3}\) synthase, R24 co-precipitated caveolin (Fig. 9).

**DISCUSSION**

In the present study, we demonstrated that a monoclonal antibody to ganglioside \(G_{D3}\), R24, co-immunoprecipitates Src (Fig. 8, C and F, respectively). This discrepancy probably derives from differences in the protein/detergent ratio during the initial homogenization, as has recently been pointed out (35), because most of Lyn and about 70% of Src from the transfectants in 10 150-mm dishes were present in the light scattering band (data not shown). In the CST cells, caveolin (a marker protein of caveolae) was also present in the low density fraction (Fig. 8G). Caveolin was not detected in homogenate of cerebellum (data not shown).

**Immunoprecipitation of Caveolin with R24**—We investigated whether R24 immunoprecipitated caveolae of CHO cells. In CHO cells transfected with \(G_{D3}\) synthase, R24 co-precipitated caveolin (Fig. 9).
family tyrosine kinase Lyn from Triton X-100 extracts of rat brain and primary cerebellar cell cultures. This suggests that there is a specific association of Lyn with GD3 on rat brain cell membrane. The main region of the GD3-Lyn association in brain may be cerebellar granule cells. Indeed, both Lyn mRNA and protein are predominantly expressed in the cerebellar granule cells, as has been shown in in situ hybridization and immunohistochemistry (31, 36). The same area expresses GD3 synthase and synthesizes GD3 (19, 37–40); the latter can be detected on the surface of rat primary cerebellar cells by flow cytometric analysis (this study).

Binding of R24 to GD3 activated Lyn and induced rapid tyrosine phosphorylation of several proteins in rat primary cerebellar cell cultures. This suggests that the GD3-Lyn association is not an artifact of detergent extraction and that GD3 could mediate transmembrane signaling in rat cerebellar granule cells via Lyn. Similar observations were reported for human peripheral T cells, where treatment with R24 led to T cell activation associated with rapid tyrosine phosphorylation of several substrates including phospholipase Cγ, as well as to phosphatidylinositol turnover, calcium flux, Ras activation, cell proliferation, and cytokine secretion. The phosphatidylinositol turnover and cell proliferation can be blocked by a tyrosine kinase inhibitor (41–43), suggesting that GD3-mediated tyrosine kinase activation has an important role in the activation of T cells. The association of Src family kinase Lyn with gangliosides has also been reported for rat basophilic leukemia RBL-2H3 cells (44–46). In this system, a monoclonal antibody AA4, which recognizes α-galactosyl derivatives of ganglioside GD3b, on RBL-2H3 cells, co-precipitates Lyn and the IgE receptor. Binding of AA4 leads to activation of Lyn, and subsequent increase in tyrosine phosphorylation of several substrates including phospholipase Cγ1, phosphatidylinositol turnover, calcium flux, activation of protein kinase C and, ultimately, morphological change of RBL-2H3 cells. The effects induced by AA4 on Lyn are similar to those seen following Lyn activation through the IgE receptor. These data suggest that the interaction of Src family kinase with ganglioside could play a role in receptor-mediated signal transduction.

Binding of R24 to GD3 in rat primary cerebellar cultures induced phosphorylation of MAPK at Tyr-204, which was shown to be critical for the enzymatic activation of MAPK. The time course of Lyn autophosphorylation and the phosphorylation of MAPK suggests that Lyn activation is one of the upstream inputs of the MAPK cascade. Lyn is known to be necessary for activation of the MAPK cascade in the G-protein–coupled receptors signal transduction (47). In pheochromocytoma cell line PC12, nerve growth factor promotes neural differentiation and induces MAPK activation, which is necessary for the differentiation (48). Therefore, GD3 might modulate neural development of the cerebellum via the MAPK signaling pathway.

Initially, GD3 and Lyn were detected in the Triton X-100 extracts of rat cerebellum cells. By subcellular fractionation, using a sucrose gradient centrifugation, they were found in membranal complexes called DIGs. DIG is a membrane domain, which appears to be present in all mammals and yeast (34, 49). Two types of DIG structures have been described: caveolar and non-caveolar domains. Caveolae (50–100-nm invaginations of the plasma membrane; Ref. 50) are thought to be built up around the DIGs with caveolin (34), a coat protein of caveolae (51, 52). In cells expressing caveolin, the DIG fraction contains caveolae. DIG is also referred to as caveolea-like domain (53). DIGs and caveolae have many properties in common. (i) They are resistant to dissociation by detergents like Triton X-100. The insolubility has been attributed to the lipid components, glycosphingolipid and cholesterol (54, 55). (ii) They are isolated as a low density light-scattering band by sucrose density gradient analysis. (iii) They are enriched in glycosphingolipid, sphingomyelin, cholesterol, glycosyl phosphoryl-CoA-independent protein, and signaling molecules like the Src family kinases and G-proteins (30, 56–58). This observation led to the idea that the DIGs mediate signal transduction (59–61). Localization of epidermal growth factor-stimulated Ras/Raf-1 interaction to caveolae membrane suggests that it is the initiation site of the MAPK cascade (62). Caveolin binding negatively regulates the auto-activation of Src family kinases (63). Recently, it was reported that Src family kinase Lek was selectively regulated in the DIGs of T cell lymphoma (64). IgE receptor-mediated activation of RBL-2H3 cells caused recruitment of Lyn to the DIGs (65). Although caveolae structures have been seen in thin-section images of neuronal cells (66), isoforms of caveolin have not been found yet in the nervous system. However, the DIGs have been isolated from the nervous system. Neuronal DIGs are enriched in Src family kinase Fyn, Yes, and heterotrimeric G-protein, Ras, nerve growth factor receptor Trk B and p75 (53, 67, 68). In the present study, we noted the following. (i) Lyn of a rat cerebel-

**Fig. 8.** Sucrose density gradient analysis of Lyn in a rat cerebellum and CHO transfectants. Rat cerebellum (A–C) and CST transfectants with Lyn cDNA (D, E, and G) or Src cDNA (F) were lyed in Triton X-100 and linear sucrose gradients (5–30%) were formed over them. Nine fractions were collected from 5% to 30% after centrifugation. Fraction 10 was collected from 40% sucrose. Protein profile (A and D) and immunoblotting with anti-Lyn (B and E), anti-Src (C and F), and anti-caveolin (G) in each fraction.
lum was detected in the low density fraction. Lyn and caveolin of CHO transfectants were also detected in the same fraction. (ii) Autophosphorylated Lyn precipitated with R24 (p53/56) was greatly reduced by solubilization of brain membrane with 60 mM octylglucoside. This observation is consistent with the fact that the DIGs are dissociated by octylglucoside. It has been attributed to the resemblance of the detergent to glycolipid (30). (iii) Although the DIGs are Triton X-100-insoluble, they are detected in postnuclear supernatants from cells extracted by Triton X-100 or Nonidet P-40 (69, 70), which are used for immunoprecipitation in this study. (iv) R24 precipitated Lyn, but not Src in CST cells transfected with either Lyn or Src cDNA. This could be caused by the absence of Src from DIGs. It has been reported that both palmitoylation and myristoylation of proteins are critical for localization to the DIGs (71). Lyn, which is modified by palmitoylation and myristoylation, preferentially localizes to the DIGs, but Src, which is modified only by myristoylation, does not. Lyn, which undergoes both palmitoylation and myristoylation, was co-immunoprecipitated by R24 in CST cells transfected with Fyn cDNA. Furthermore, R24 immunoprecipitated caveolin in CST transfectants. This finding suggests that caveolin is also a ganglioside-binding protein (72). Therefore, R24 may precipitate the DIGs or caveolae-like domains containing Lyn of rat brain cell membranes.

Cerebellar granule cells proliferate at the external granular layer, migrate to the internal granular layer, and undergo neurogenesis and synaptic formation of an axon with a dendrite of a Purkinje cell (73). During this development, a change takes place in the ganglioside content of cerebellar granule cells (74). GD3 is the major species in immature granule cells. GD3 is the subject of future research.

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