Fucose-containing glycolipids are stage- and region-specific antigens in developing embryonic brain of rodents

(cerebral cortex/X-determinant glycoconjugates)

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ABSTRACT A monoclonal antibody (against 7A antigen), raised against a homogenate of embryonic rat forebrain, marks the distribution of region- and developmental stage-specific cell-surface antigens in the mammalian central nervous system. In the mouse, immunocytochemical staining revealed that 7A antigen is expressed almost exclusively in germinal layers of the cerebral cortex beginning as early as day 11 of gestation and becomes undetectable by birth. The typical staining is seen at embryonic days 13–15, in which only the ventricular and the subventricular zones of the cerebral cortex are intensely labeled, whereas most other areas of the brain are unlabeled. Tissue culture studies indicate that the 7A antigen is localized at the plasma membrane. Molecular species possessing the antigen are identified as neutral glycolipids that contain the X-determinant trisaccharide:

This glycoconjugate is expressed in embryonic brains of many mammalian species (mouse, rat, rabbit, calf, and human) and may be a cell-surface component important in normal development of the central nervous system.

The nervous system develops through a sequence of cellular events, including cell proliferation, migration to particular sites, and various states of morphological differentiation leading to the establishment of the specific afferent and efferent connections. A general hypothesis is that these events are regulated by the interaction of molecules expressed on cell surfaces, in the extracellular matrix, or by diffusible substances. There is increasing evidence that certain glycoproteins are involved in specific cell–cell interactions in the development of the peripheral nervous system. For example, the glycoproteins N-CAM (neural cell adhesion molecule) and fibronectin undergo reciprocal changes in expression in association with the migration of neural crest cells and their aggregation into ganglia (1, 2). *In vitro* experiments suggest a causal role of N-CAM in promoting cell–cell adhesion (3, 4) and for fibronectin in stimulating migration (5). Temporal and spatial changes in the expression of these two glycoproteins in the central nervous system (CNS) suggest that they may play a comparable role in the control of migration and settling of granule cells of the cerebellum (6).

Glycolipids are an alternative candidate for glycoconjugate molecules mediating developmental interactions. Expression of glycolipids also appears to correlate with differentiation of embryonic cells and tumor cells in culture (7). In the development of the nervous system, however,

detailed studies of glycolipid expression are just beginning to emerge. Studies of acidic glycosphingolipids (gangliosides) have shown that there are definite correlations between the expression of gangliosides GM3 and GD3 (monosialo and disialo derivatives of lactosylceramide, respectively) and CNS development (8). In the developing cerebellum, ganglioside GM1 increases during development of granule cell neurons (9). Results using anti-ganglioside monoclonal antibodies for immunohistochemical studies of mammalian CNS suggest that both GD3 ganglioside (10) and D1-1 ganglioside (11), which contains *O*-acyl sialic acid (33), are associated with undifferentiated cells in early postnatal cerebellum. However, the significance of these correlations is not clear, and the function of gangliosides in the CNS has remained ambiguous. Some recent studies indicate that gangliosides may be involved in cell–cell interactions in the CNS. GM2 has been proposed to play a role in adhesive interactions in the chicken retino-tectal projection (12).

In contrast to these promising studies of developmental changes in ganglioside expression, very few neutral glycolipids other than galactosylceramide have been identified in embryonic or adult CNS, either chemically or immunologically (13, 14). In this paper, we report that a monoclonal antibody produced against embryonic brain, which recognizes neutral glycolipids that express the X-determinant trisaccharide, labels the surface of a distinct class of cells in the developing cerebral cortex and reacts with few noncortical cells in the embryonic brain. These glycolipids are expressed mainly on proliferating cells, which are precursors of both neurons and glia, but not on migrating or postmigratory cells. This suggests that the expression of these glycolipids may modulate the cell–cell interactions in the normal developmental processes in particular regions of the brain.

MATERIALS AND METHODS

Hybridoma Production. BALB/c mice were immunized three times at 2-wk intervals with the homogenate obtained from embryonic rat forebrain. Three days after the final injection, mice were sacrificed and spleen cells were fused with the myeloma cell line (NS-1) as described (15). Supernatants from 96-well plates were screened on tissue sections of embryonic brain prepared as described below. A monoclonal antibody against 7A antigen was obtained. The subclass was identified as IgM by using subclass-specific secondary antibody.

Materials. Sprague–Dawley rats and C₃B₆ mice were used. Mice were mated in our colony, and timed-pregnancy rats were obtained from Charles River Breeding Laboratories. The day of detection of a sperm plug is determined as gestation day [embryonic day 0 (E0)]. For immunocyto-

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Abbreviations: E, embryonic day; P, postnatal day; CNS, central nervous system; SSEA-1, stage-specific embryonic antigen 1.

chemical analysis, E9, E10, E11, E13, E15, E17, E19 (postnatal day 0; P0), P4, P7, P14, P21, and adult mice and E13, E15, and E17 rats were used. For glycolipid analysis, E11 mouse and E15–17, P0, and P14 rats were used. Embryonic brains of rabbits and calves were also used for the immunochemical analyses.

Immunocytochemistry. Pregnant animals were anesthetized with ether and decapitated. Embryos were removed from the uterus and quickly frozen by dipping in 2-methylbutane cooled by dry ice. Serial 10- μ m sections of the whole embryo were prepared by cryostat, mounted on gelatin/chrome alum-coated slides, and dried thoroughly. Mounted sections either were left unfixed or were postfixed with 4% paraformaldehyde (10 min) or acetone (5 min at RT). After preincubation with 3% normal goat serum in phosphate-buffered saline (P_i /NaCl) at pH 7.4, sections were incubated with antibody 7A (1 mg/ml; 1:10 dilution in P_i /NaCl at 4°C overnight). After sections were washed, they were incubated with peroxidase-conjugated anti-mouse IgM (Boehringer Mannheim) diluted 1:50 in P_i /NaCl for 30 min at RT. Sections were then incubated with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB) in P_i /NaCl containing 0.002% of H_2O_2 for 20 min, washed, and exposed to 0.01% osmium tetroxide for 3 min. Sections were dried, dehydrated, and mounted with Eukit. In some cases a light counterstain with 0.5% cresyl violet was done.

Cell Culture. Brains obtained from E13–15 rats were separated into three parts—telencephalon, diencephalon, and brain stem. Cells were dissociated with 0.025% trypsin (GIBCO), plated onto rat-tail collagen or poly(L-lysine)-coated plastic dishes with Ham's F-12 medium supplemented with penicillin/streptomycin, L-glutamine, 10% fetal calf serum, and vitamins as described (16). Cells were maintained up to 3 wk.

Cell Lines. The following cell lines are maintained in culture. Neuroblastomas NB2A, N1E, and N18; C6 glioma; and teratocarcinomas 402AX, Ter C, and F9.

Immunocytochemistry on Cultured Cells. Cultures were incubated with anti-7A antibody diluted 1:10 with Hanks' salt solution at 37°C for 30 min. Cultures were washed with the salt solution and fixed with acetone/ethanol, 1:1 (vol/vol), at –20°C for 10 min. Cultures were then incubated with fluorescein isothiocyanate-conjugated anti-mouse IgM (diluted 1:50 with P_i /NaCl) for 30 min at RT, washed, and observed under epifluorescent illumination. Some cultures were prefixed with 4% paraformaldehyde before the incubation with anti-7A antibody.

Western Blotting. Brain homogenate from E13–15 or P14 rats was electrophoresed with NaDodSO₄ gel chromatography and blotted onto nitrocellulose paper as described (17). The nitrocellulose paper was incubated with 3% normal goat serum and then with anti-7A antibody at 4°C overnight, washed, and incubated at RT for 2–3 hr with peroxidase-conjugated anti-mouse IgM (1:1000 in P_i /NaCl). The reaction product was visualized with 3,3'-diaminobenzidine.

Glycolipid Extraction and Analysis. The chloroform/methanol extracts were dried under nitrogen, resuspended in distilled water, and desalted on Bond-Elut cartridges (18) (Analytical Chemicals, Harbor City, CA). The desalted glycolipids were chromatographed on DEAE-Sephadex A-25 (Pharmacia) to separate neutral glycolipids from gangliosides (19). Neutral glycolipids were treated with 0.6 M methanolic NaOH for 1 hr at RT, neutralized with 0.5 M HCl, and desalted. The desalted neutral glycolipids were chromatographed on high-performance TLC plates (Merck) in chloroform/methanol/water, 60:35:8 (vol/vol).

Radiolabeling. Cell cultures containing $\approx 10^7$ cells were incubated for 18 hr with 100 μ Ci (1 Ci = 37 GBq) of [³H]fucose (New England Nuclear). Cells were then harvested and extracted as above. TLC plates containing radio-

actively labeled glycolipids were exposed on Ultrafilm (LKB) for 4–14 days at RT.

Immunochromatography. Neutral glycolipids and gangliosides were chromatographed on aluminum high-performance TLC plates (Merck) in chloroform/methanol/water, 60:35:8 (vol/vol), dried, and then dipped in 0.05% poly(isobutyl methacrylate) in hexane as described by Brockhaus *et al.* (20). The plates were then soaked in P_i /NaCl containing 1% bovine serum albumin for 2 hr before exposure to anti-7A antibody for 2 hr at 4°C. After the plates were washed in P_i /NaCl, they were exposed to goat anti-mouse IgM conjugated to horseradish peroxidase for 2 hr at 4°C. The plates then were washed in P_i /NaCl and developed with 33 mM 4-chloronaphthol in 0.02 M Tris-HCl buffer containing 20% methanol and 0.025% H_2O_2 .

Immunoprecipitation. Immunoprecipitation was carried out as described (21). Briefly, 10^7 cells were labeled with 400 μ Ci of [³⁵S]methionine for 2 hr at 37°C. After being washed, the cells were lysed in 0.01 M Tris in P_i /NaCl containing 1% Trasylol, 0.1 mM phenylmethylsulfonyl fluoride, 0.05% NaDodSO₄, and 1% Triton X-100. Lysates containing 10^6 cpm of radioactivity were incubated with 50 μ l of purified anti-7A antibody overnight at 4°C, followed by 50 μ l of rabbit anti-mouse IgM and 50 μ l of Pansorbin (Calbiochem–Behring). After the precipitate was washed several times, it was boiled for 2 min in sample buffer. After removal of the Pansorbin, the supernatant was electrophoresed on NaDodSO₄/polyacrylamide gels (22). Immunoreactive bands were visualized by autoradiography.

RESULTS

Distribution of 7A in the Embryonic Brain. Immunocytochemical studies revealed that the 7A antigen is differentially distributed in the rodent brain from early stages of histogenesis. In the mouse, stages E9 through birth (E19), P21, and adult were examined. Temporal changes in the distribution of the antigen in embryonic mouse brain are summarized in Fig. 1. The most outstanding feature of the distribution of this antigen is that it is localized almost exclusively in the cerebral cortex from day E11, particularly in cells of the germinal zones. This staining had a mesh-like appearance which suggests an association with either the plasma membrane or extracellular space (Fig. 2F). At day E9, the surface of the neural tube showed immunoreactivity. At E10, staining appeared in the lateral wall of the forebrain, the midbrain, and the central portion of the spinal cord. In the period from E11 to E13, the entire lateral wall of the cortical anlage was strongly positive (Fig. 2 A–C), but not much staining was present in the medial wall. There was a rostro-

FIG. 1. Summary diagram of temporal expression of the 7A antigen in the developing nervous system of the mouse. The width of the lines corresponds to intensity of tissue reactivity demonstrated by immunocytochemistry.

FIG. 2. Immunohistochemical localization of 7A antigen in the mouse embryonic brain. (A) Camera lucida drawings of successive levels (a-e, shown in f) of a stage E11 mouse brain. Filled areas are immunoreactive. (Bar = 1 mm.) (B) A section through the cortex and the diencephalon (di) of E13 mouse. Only the cortex is strongly immunoreactive. (Bar = 500 μ m.) (C) High magnification of B. The entire cortical wall is positive. (Bar = 200 μ m.) (D) Coronal section of E17 mouse forebrain. The strongest reactivity is seen in the cortex and on a small hypothalamic region. (Bar = 500 μ m.) (E) Higher magnification of cortex in D. Only the ventricular (vz) and subventricular zones (svz) are strongly positive. iz, Intermediate zone; cp, cortical plate. (Bar = 200 μ m.) (F) High magnification of positive regions in D. The asterisk marks the ventricular cavity. (Bar = 10 μ m.) Configuration of the tissue is outlined by dotted lines.

caudal gradient in the immunoreactivity in the cortex. The caudal portion was more intensely labeled than was the rostral portion, and the olfactory bulb was devoid of labeling (Fig. 2A, level e) at these stages. Two small discrete areas, one in the hypothalamic region (Fig. 2A, level d) and the other in the ventral wall of the inferior colliculus (Fig. 2A, level a), also were labeled. No stain was seen in most of the diencephalon, in the rhombencephalon, or in the spinal cord. In the interval from E15 to E17, after the neo-cortical plate had formed, dense staining was restricted to the ventricular and the subventricular zones in the cortex (Fig. 2D and E). Only weak staining was seen in the deeper half of the intermediate zone. At this stage, the subiculum and the cingulate cortex were immunoreactive but the entorhinal cortex was not. Portions of the hypothalamus and inferior colliculus were still stained. Weak staining began to appear in the medulla, thalamus, and hippocampus by E17 (Fig. 2D). At E19 (P0) in the mouse, staining in the cortex disappeared entirely. Thereafter, less discrete, more diffuse staining of the extracellular compartment began to appear in the thalamus, basal ganglia, medulla, and spinal cord. This reactivity increased and persisted up to the third postnatal week and then gradually decreased. In the rat, the patterns of emergence of immunoreactivity were basically similar to those in the mouse. However, the anlagen of the basal ganglia and the neural retina were strongly immunoreactive from E13 in this animal.

Tissue Culture. The immunoreactive cell types and subcellular localization of antigen were assessed by using primary tissue culture obtained from rat embryonic brain. Staining of both living and prefixed cells confirmed that immunoreactivity is localized in the plasma membrane. In cultures prepared from the telencephalon of the E13 rat, about 80–90% of the total cell population was stained with anti-7A antibody 1 hr after dissociation with trypsin and plating (Fig. 3 A and B). In cultures prepared from the diencephalon or the brainstem, by

contrast, only 20–30% of cells were immunoreactive (Fig. 3 C and D). However, after 1–2 wk in culture, only a low percentage of telencephalic cells were immunoreactive (10–30%). Most but not all cells with neuronal features, such as having extensive neurite elaboration, did not show staining. Most immunoreactivity was associated with large flattened cells. Double staining with anti-vimentin antibody (provided by R. Hynes) in 1-wk-old cultures showed that most of the anti-7A-positive cells are vimentin negative. Thus, the selectivity of expression of 7A antigen among cortical cells and its loss with maturation is characteristic of

FIG. 3. Immunofluorescence staining by 7A of living cells in tissue culture prepared from E13 rat forebrain (A and B) and diencephalon (C and D) 1 hr after being plated on a polylysine-coated dish. The same staining pattern is observed with the cells prefixed with 4% paraformaldehyde. (A and C) Immunofluorescence. (B and D) Phase-contrast pictures of the same microscopic field.

both *in vivo* and *in vitro* preparations. The high frequency of initial staining in telencephalic cultures suggests that the antigen is expressed in both glial and neuronal precursors.

Cell Lines. Among neuroblastoma (NB2A, N1E, N18), C6 glioma, and teratocarcinoma (402AX, TerC, F9) cell lines, anti-7A antibody reacted only with undifferentiated F9 by immunocytochemistry. However, anti-7A did not react with any glycolipid extracted from F9 cells (data not shown).

Distribution in the Nonneuronal Tissue in Embryo. In early embryonic stages of the mouse (E9–E11), intense immunoreactivity was found in auditory vesicles and the epidermis of the head portion. At E13–17, besides these areas, portions of bronchial epithelium, stomach epithelium, and nasal epithelium were stained. At E17, ocular conjunctiva were also immunoreactive.

Glycoconjugate Analysis. The biochemical nature of the molecule(s) that reacts with anti-7A antibody was examined. Reactivity against proteins was assessed by electrophoretic immunoblotting (17) and immunoprecipitation (21) on homogenates obtained from E15–17 and P14 rat brain. These procedures did not provide clear evidence of the presence of immunoreactive proteins. However, when anti-7A was allowed to react on thin-layer chromatograms containing either neutral glycolipids or acidic glycolipids (gangliosides) isolated from E15 rat brain, three immunoreactive neutral glycolipid bands were recognized. Gangliosides and neuraminidase-treated gangliosides were not reactive with anti-7A (data not shown). Each immunoreactive neutral glycolipid was split into two or three bands (Fig. 4A), probably the result of the heterogeneity within the ceramide portion of these molecules bearing the 7A epitope; each doublet in Fig. 4A is numbered to show identity on subsequent chromatograms. Quantitative changes in the neutral glycolipids that occurred in brain during embryonic and postnatal stages are shown in Fig. 4C. During this develop-

mental time frame, galactosylceramide increased dramatically, while the more complex glycolipids decreased or disappeared. A postnatal decrease in immunoreactive glycolipids was also seen. These anti-7A-reactive glycolipids were detected as early as day E11 in the mouse. In the rat, high concentrations were present in the E15–17 interval, decreased after P0, and became nearly undetectable at P14 (Fig. 4D).

To determine the carbohydrate structure of these glycolipids, [3 H]fucose labeling of dissociated E15–17 brain was carried out. Significant incorporation of fucose into three glycolipids (numbered 1–3) that are chromatographically identical to the anti-7A antibody-reactive glycolipids is shown in Fig. 4B. Immunoreactivity of anti-7A is the strongest with the largest fucoglycolipid (no. 3) and weakest with the smallest one (no. 1). This could represent either a cross-reactivity or a preference of the antibody for longer-chain structures as has been described (23). To characterize the carbohydrate structure of anti-7A glycolipids further, immunochromatography was carried out against neutral glycolipid extracted from E15–17 rat brain by using a panel of known fucose-reactive blood group antibodies, including anti-H (24), anti-Le^a (Lewis^a blood group) (25) and anti-SSEA-1 (stage-specific embryonic antigen 1) (26). The reactivity of anti-7A antibody with each of these blood group antigens was also tested. From these studies it is clear that anti-SSEA-1, which is known to react with X-determinant glycoconjugates, reacted with the same fucose-containing glycolipid as did anti-7A, and further that anti-7A reacts with III³- α -fucosyl-lactoneotetra glycosylceramide (gift of V. Ginsburg, National Institutes of Health), which is an X-determinant glycolipid (data not shown).

DISCUSSION

The most distinctive feature of the antigen is its relatively restricted distribution to the cortical anlage from early embryonic stages to the time of birth. The time course of staining in the cortex suggests that there are two (or more) types of molecules recognized by anti-7A antibody. Staining appears at an early embryonic stage and disappears at birth (Fig. 1). However, cellular reactivity appears again postnatally in the cortex, at a time when anti-7A-reactive glycolipid has decreased significantly and is probably no longer expressed in the cortex. This indicates that the antibody is probably recognizing multiple molecular species: glycolipid prenatally and glycoprotein or proteoglycan postnatally, all of which may share the same carbohydrate antigenic determinant. Although it is difficult to distinguish each of these glycoconjugates by immunocytochemical staining, immunoreactivity in the embryonic cortex is likely to be mostly due to glycolipid since it remains after trypsin treatment (Fig. 3). In other areas of the brain, identification of the molecular species of the antigenic determinant may be more difficult. As shown in Fig. 1, a small portion of both the hypothalamus and the inferior colliculus are immunoreactive at day E11. This reactivity continues throughout prenatal and postnatal development. The early staining in these noncortical areas is probably partly due to glycolipid expression since it is resistant to trypsin treatment (Fig. 3). In later stages (around day E17), when diffuse reactions appear in the hippocampus, thalamus, and medulla, it is possible that other X-determinant glycoconjugates are also expressed. The reactivity of the nonneuronal tissue has not been chemically studied to determine whether it is also due to glycolipid.

From the immunochemical comparison of anti-7A and anti-SSEA-1 antibodies, it is apparent that both antibodies recognize the same carbohydrate moiety, the X-determinant. Antibodies to SSEA-1 were first produced by immunization with the teratocarcinoma cell line F9. When first described,

FIG. 4. (A) Thin-layer chromatogram of embryonic rat brain neutral glycolipids as detected by immunochromatography with anti-7A antibody (left lane) and orcinol spray (center lane). Standards are in the right lane. (B) Thin-layer chromatogram (lanes a) and autoradiogram (lanes b) of [3 H]fucose-labeled E15–17 rat brain neutral glycolipids. Standards are [3 H]galactose-labeled. (C and D) Thin-layer chromatograms of the developmental profile of rat brain neutral glycolipids as detected by orcinol spray (C) and immunoreactivity with anti-7A antibody (D), showing the quantitative decrease in complex neutral glycolipids between stages E15–17 and P14. Each lane on both chromatograms represents glycolipids extracted from 25 mg (wet weight) of tissue. Standards are galactosylceramide (CMH), lactosylceramide (CDH), trihexosylceramide (CTH), globotetraosylceramide (GLOB), and ganglio-tetraosylceramide (AGM1). The three major fucose-containing glycolipids are numbered on each chromatogram to indicate their identity as detected by each procedure.

SSEA-1 was believed to be a marker for differentiation of the mouse preimplantation embryo (26). The antigen appeared at the 8-cell stage, was maximally expressed in the morulae, and disappeared at the blastocyst stage. The carbohydrate specificity of anti-SSEA-1 antibody was determined several years later (27). Only recently was a group of polydisperse glycoproteins, but not glycolipids, from F9 cells found to react with anti-SSEA-1. In the meantime, it was found that anti-SSEA-1 reacted not only with mouse preimplantation embryonic tissue but also with other fetal and adult mouse and human tissues, including various tumors (28). Recent studies now have shown that glycolipids from human adenocarcinoma (29) and granulocytes (21, 30) possess the X-determinant structure and react with anti-SSEA-1 or similar antibodies. To date, though, the molecular nature of the X-determinant glycoconjugates in embryonic tissue remains unknown.

Although anti-7A and anti-SSEA-1 antibodies recognize similar carbohydrate structures, a comparison of their cellular reactivities in neuronal and nonneuronal tissues has revealed a difference. Lagenaur *et al.* (31) reported that anti-SSEA-1 reacted weakly with germinal cells of embryonic mouse brain. However, the reaction was not specifically localized, as is the case for anti-7A. In postnatal cerebellum (31), anti-SSEA-1 stains the external granule cell layer and prospective white matter *in vivo* and astrocytes *in vitro*. Anti-7A shows a similar staining pattern in the postnatal cerebellum, but the reaction is weak compared to the embryonic cortex. Other differences of the immunoreactivity are seen in embryonic nonneuronal tissue. Anti-7A and anti-SSEA-1 both react with epidermis and auditory vesicles. Anti-7A also reacts with nasal epithelium, ocular conjunctivum, bronchial epithelium, and stomach epithelium, but it is not clear whether these tissues are also anti-SSEA-1 positive (32). The reactivity with F9 seems the same for both antibodies. Thus, the tissue distributions of anti-7A and anti-SSEA-1 are similar but not identical. These differences in tissue reactivity of the two antibodies may be ascribed to slight differences in either specificity or binding affinity but also may be due to differences in staining techniques.

Developmentally regulated gangliosides that have been described previously have not been extensively characterized immunocytochemically in embryonic brain. In the postnatal cerebellar cortex (10, 11), antibodies to both ganglioside GD3 and D1-1 stain the external granule cell layer. Anti-D1-1 also stains the germinal zones of the embryonic forebrain. Anti-7A recognizes both of these areas; however, the mapping with anti-7A antibody at various stages of development reveals that the antigen is distributed in a very restricted manner to the cortex in the CNS. A further study with these ganglioside antibodies might potentially reveal specific distribution of the antigens.

The work reported here demonstrates that there is a group of fucose-containing glycolipids that is expressed in relatively high concentrations in embryonic rodent brain but disappears shortly after birth (see Fig. 4). These glycolipids react by ELISA and immunochromatography equally well with anti-SSEA-1 and anti-7A antibodies. These glycolipids are present in the cortical layers containing proliferative and premigratory cells but not in layers containing migratory and postmigratory cells. We recently have detected these glycolipids in embryonic brain of a number of vertebrates including chicken, rabbit, calf, and human. The existence of X-determinant glycolipids in embryonic and early postnatal tissue from the CNS and the restricted distribution of 7A antigens suggests the possibility that these glycolipids may be specifically involved in normal development of the embryonic cerebral cortex. The function of neutral or acidic glycolipids in these germinal cells is poorly understood but it

seems likely that they may mediate a role in cell proliferation or cell-cell interactions.

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