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Expression of neolactoglycolipids: sialosyl-, disialosyl-, *O*-acetyldisialosyl- and fucosyl- derivatives of neolactotetraosyl ceramide and neolactohexaosyl ceramide in the developing cerebral cortex and cerebellum

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The following neolacto glycolipids were identified and their developmental expression was studied in the rat cerebral cortex and cerebellum: Fucα1-^{3}IIInLcOse$_4$Cer,Fucα1-^{3}VnLcOse$_6$Cer and (Fuc)$_2\alpha$1-^{3}III,^{3}VnLcOse$_6$Cer, as well as acidic glycolipids, NeuAcα2-^{3}IVnLcOse$_4$Cer [nLM1], (NeuAc)$_2\alpha$2-^{3}IVnLcOse$_4$Cer [nLD1], *O*-acetyl (NeuAc)$_2\alpha$2-^{3}IVnLcOse$_4$Cer [OAc-nLD1] and their higher neolactosaminyl homologues NeuAcα2-^{3}VInLcOse$_6$Cer [nHM1] and (NeuAc)$_2\alpha$2-^{3}VInLcOse$_6$Cer [nHD1]. These glycolipids were expressed in the cerebral cortex only during embryonic stages and disappeared postnatally. This loss was ascribed to the down regulation of the synthesis of the key precursor LcOse$_3$Cer which is synthesized by the enzyme lactosylceramide: *N*-acetylglucosaminyl transferase. On the other hand in the cerebellum, these glycolipids increased with postnatal development due to increasing availability of LcOse$_3$Cer. In the cerebellum, only nLM1 and fucosyl-neolactoglycolipids declined after postnatal day 10–15, perhaps due to regulation by other glycosyltransferases. Also, in the cerebellum, nLD1 and nHD1 were shown to be specifically associated with Purkinje cells and their dendrites in the molecular layer and with their axon terminals in the deep cerebellar nuclei, similar to other neolactoglycolipids shown previously.

Keywords: neolactoglycolipids in cerebral cortex, neolactoglycolipid in cerebellum, Purkinje cells, Le^x antigens, neural development

Introduction

Lacto series of glycosphingolipids are derived from the core structure lactotriosylceramide (LcOse$_3$Cer, See Fig. 1). They are subdivided into two groups based upon: lactoseries (Type 1) structure, in which a galactose is added to the terminal GlcNAc of LcOse$_3$Cer in a β1-3 linkage to form lactotetraosylceramide Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ1-1ceramide (LcOse$_4$Cer), and upon neolacto (Type 2) structure, in which a galactose is added to terminal GlcNAc of LcOse$_3$Cer in a β1-4 linkage to form neolactotetraosylceramide (nLcOse$_4$Cer). The biosynthetic pathway of several nLcOse$_4$Cer derived glycolipids which are expressed in the nervous system is shown in Fig. 1 Previously, we and others have reported that several neolactoglycolipids derived from nLcOse$_4$Cer (see Table 1) are expressed in the mammalian cerebral cortex only during the neonatal period and disappear in the postnatal and adult cortex [1–9]. In contrast, the expressions of these glycolipids in the cerebellum and peripheral nervous system increase with postnatal development [4–6, 8]. Thus SSEA-1/7A antibody reactive Le^x

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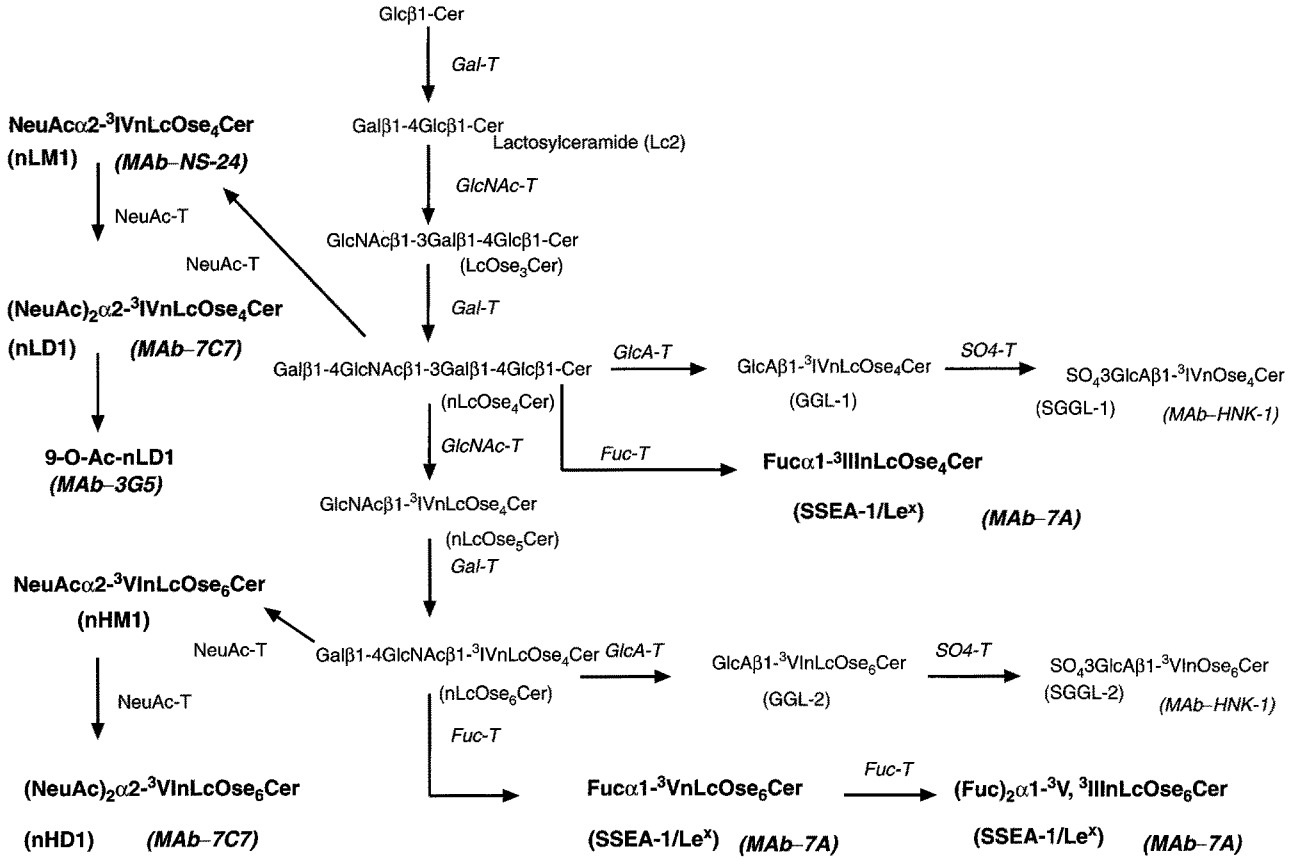

Figure 1. Biosynthesis of neolactoglycolipids. The glycolipids studied in this manuscript are identified with bold letters and their nominal abbreviations are given in parenthesis. The names of the monoclonal antibodies (MAb) which react with the corresponding lipids are also given in parenthesis.

antigens are expressed on the premigratory and rapidly proliferating neuroblasts near the ventricular zone in the cerebral cortex of embryonic rodents and are down regulated and disappear with development in postnatal animals [3]. Whereas, antibody HNK-1 reactive sulfoglucuronyl glycolipids (SGGLs) and other HNK-1 antigens are specifically expressed on the post-migratory neuronal cells in the embryonic cerebral cortex in the molecular layer (Layer I) and subplate (Layer III) and disappear postnatally [2]. The temporal and stage-specific expressions of these glycolipids in the nervous system as well as other results have implicated them to play important roles in cell recognition, cell growth and differentiation (reviewed in [10]). It has been now recognized that the sulfoglucuronyl carbohydrate is involved in organization of early neuronal settling and axonal outgrowth patterns and it acts as a ligand for neural cell interactions [11–13].

Although we have previously studied the precise developmental expression of SGGLs and their precursors $LcOse_3Cer$, $nLcOse_4Cer$ and $nLcOse_6Cer$ in rat cerebral cortex and cerebellum [7, 8, 14, 15], the pattern of expression of other neolactoglycolipids was not examined. Here, we report the developmental expression of SSEA-1/7A reactive Fucα1-^{3}IIInLcOse$_4$Cer, Fucα1-^{3}VnLcOse$_6$Cer and (Fuc)$_2$α1-^{3}III,^{3}VnLcOse$_6$Cer, as well as acidic glycolipids, NeuAcα2-^{3}IVnLcOse$_4$Cer [nLM1], (NeuAc)$_2$α2-^{3}IVnLcOse$_4$Cer [nLD1], *O*-acetyl (NeuAc)$_2$α2-^{3}IVnLcOse$_4$Cer [OAc-nLD1] as well as their higher lactosaminyl homologues NeuAcα2-^{3}VInLcOse$_6$-Cer [nHM1] and (NeuAc)$_2$α2-^{3}VInLcOse$_6$Cer [nHD1], in the cerebral cortex and cerebellum of rat.

Materials and methods

Sprague-Dawley albino rats of various ages, including timed pregnant dams, were purchased from Taconic Farms (Germantown, NY). Monoclonal antibodies (MAb) 7A and 7C7 were gifts from Drs Yamamoto and Schwarting [3]. Antibody 3G5 ascites fluid was a gift from Dr R. Nayak [16]. A clone for MAb 1B2 was obtained from American Type Culture Collection (Rockville, MD). The 7A-reactive kidney glycolipid standard was a gift from Dr R.H. McCluer [17]. Other reagents were obtained as previously described [5–8].

Table 1. Neolacto series of glycolipids in the developing nervous system.

Trivial name	*Structure*
$nLcOse_4Cer$	Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1 Ceramide
nLM1	NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1 Ceramide
nLD1	NeuAcα2-8NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1 Ceramide
9-*O*-Ac-nLD1	9-*O*-AcNeuAcα2-8NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1 Ceramide
$Fuc\alpha1\text{-}^3IIInLcOse_4Cer$	Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4Glcβ1-1 Ceramide
SGGL-1	$SO_4$3GlcAβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1 Ceramide
$nLcOse_6Cer$	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1 Ceramide
nHM1	NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1 Ceramide
nHD1	NeuAcα2-8NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1 Ceramide
$Fuc\alpha1\text{-}^3VnLcOse_6Cer$	Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1 Ceramide
$(Fuc)_2\alpha1\text{-}^3V,^3IIInLcOse_6Cer$	Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4Glcβ1-1 Ceramide
SGGL-2	$SO_4$3GlcAβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1 Ceramide

Lipid extraction

About 50 mg–1 g (wet wt) cerebral cortex or cerebellar tissues were pooled from different animals, lyophilized and dry weight and/or protein was determined. The dry tissues were then extracted with 20 volumes of chloroform:methanol:water (1:1:0.1 by volume) as previously described [1, 4, 8]. The extracted lipids were separated on a diethylaminoethyl (DEAE)-Sephadex A25 column into neutral lipid fraction (run through fraction) and mono-, di-, and poly-sialo ganglioside fractions eluted with 0.02, 0.08 and 0.5 M ammonium acetate in methanol, respectively [1, 4, 8]. The fractions were desalted by Bond-Elut cartridges [8] and individual fractions were analysed by high performance thin layer chromatography (HPTLC) with chloroform:methanol:aqueous 0.25% $CaCl_2$ (5:4:1, by volume) as the developing solvent, as previously described [4].

Microdissection of cerebellar layers

Frozen cerebellum from adult rat was sectioned sagitally into 15 μm sections in a cryostat at −14 °C. The sections were lyophilized and the dried sections under an inverted microscope were dissected into molecular layer, granule cell layer, white matter and an area containing deep cerebellar nuclei, using micro tools [8]. The cut layers were weighed and the lipids were extracted [8]. Approximately 5 mg of each layer was collected.

HPTLC–immunostaining and quantitation

The general method for the quantitative analysis of glycolipids from the neural tissues was as follows. A known portion of the appropriate glycolipid fraction from the DEAE-Sephadex column along with known amounts of standard glycolipid were applied to an alumina backed HPTLC plate (Merck) and the lipids were resolved by using chloroform:methanol:aqueous 0.25% $CaCl_2$ (5:4:1, by volume) as the developing solvent. The plate was then dried, coated with 0.05% poly(isobutyl methacrylate) in hexane, soaked in 1% bovine serum albumin solution and exposed to relevant antiglycolipid antibody [2]. After washing the plate with the appropriate buffer, it was exposed to the relevant horseradish-peroxidase-conjugated second antibody [2]. The immunoreactive bands were stained with 4-choronaphthol and H_2O_2 [2]. In some cases resorcinol staining of the HPTLC plate was also performed. The immunoreactive or resorcinol stained glycolipid bands on the HPTLC plates were quantified by reflectance scanning with a BioImage/Visage 110 computer-assisted video scanner (BioImage/Millipore, Ann Arbor, MI) and comparison with known amounts of standards alongside the samples on the same HPTLC plates [8].

The expression of monosialo neolactoglycolipids nLM1 and nHM1 was determined in the 0.02 M ammonium acetate fraction. The quantity of nLM1 was determined in two different ways: (1) by immunostaining of the HPTLC plate with MAb NS-24 [18] followed by quantitative analysis of the bands with known amounts of nLM1 standard from rat sciatic nerves; (2) by conversion of nLM1 to $nLcOse_4Cer$ by mild acid hydrolysis [19] followed by reaction with MAb 1B2 [20] and comparison with known amounts of standard $nLcOse_4Cer$. nHM1 was analysed by resorcinol as well as by conversion to $nLcOse_6Cer$ followed by reaction with MAb 1B2.

The expression of disialoneolactoglycolipids nLD1 and nHD1 in the 0.08 M ammonium acetate fraction were analysed by HPTLC-immunostaining with MAb 7C7 and comparison with a known amount of standard GD3, as previously described [6].

The expression of OAc-nLD1 in the disiaologanglioside fraction was by HPTLC-immunostaining with MAb 3G5 [6]. Since standard OAc-nLD1 was not available, only relative amounts, based on optical density of bands at each age, were determined. The expression of the Le^x-determinant glycolipids in the neutral lipid fraction was determined by immunostaining with MAb 7A [3, 8]. In

the case of the cerebral cortex only relative amounts based on optical density were determined, however in the cerebellum, the amounts were determined in comparison with the known amounts of 7A-reactive glycolipid from kidney [17].

Results

The biosynthetic pathway for the neolactoglycolipids expressed in the nervous system is shown in Fig. 1. The glycolipids studied in this paper are in bold letters and the antibodies with which the glycolipids specifically react are given in parenthesis.

Development expression nLM1 and nHM1

MAb NS-24 has been previously shown to be highly specific for nLM1 [18]. Two glycolipids in the monosialoganglioside fraction, isolated from embryonic rat cerebral cortex, reacted with NS-24 (Fig. 2A, lane 1). The upper band migrated slightly more slowly than standard nLM1 isolated from rat sciatic nerves [21, 22] (Fig. 2A, lane 2). The lower faintly NS-24-reactive band (shown in parenthesis) in lane 1 was the higher neolactosaminyl analogue of nLM1, i.e. nHM1. nHM1 is known to react poorly with MAb NS-24 [18]. Other glycolipids, such as NeuAcα2-^{6}IVnLcOse$_4$Cer, NeuAcα2-^{3}IVLcOse$_4$Cer (Type I), *N*-glycolylneuraminyl α-2-^{3}IV-nLcOse$_4$Cer, any of the ganglio series of gangliosides or nLcOse$_4$Cer, do not react at all with NS-24 [18].

On treatment with mild acetic acid to desialylate the terminal neuraminic acid, nLM1 and nHM1 in the monosialoganglioside fraction lost their reactivity to NS-24 and both neutral products formed were strongly reactive with MAb 1B2 (Fig. 2B) [20], which is known to react specifically with nLcOse$_4$Cer and nLcOse$_6$Cer [5, 20, 21]. Similar to nLM1, the product nLcOse$_4$Cer (Fig. 2B, lane 1) migrated slightly more slowly than standard nLcOse$_4$Cer (Fig. 2B, lane 2), whereas the product nLcOse$_6$Cer comigrated with the major upper band of the doublet of standard nLcOse$_6$Cer (Fig. 2B). These results indicate that the original gangliosides were nLM1 and nHM1 [21].

The slower migration of nLM1 (Fig. 2A, lane 1) (as well as of nLcOse$_4$Cer derived from it, Fig. 2B, lane 1)

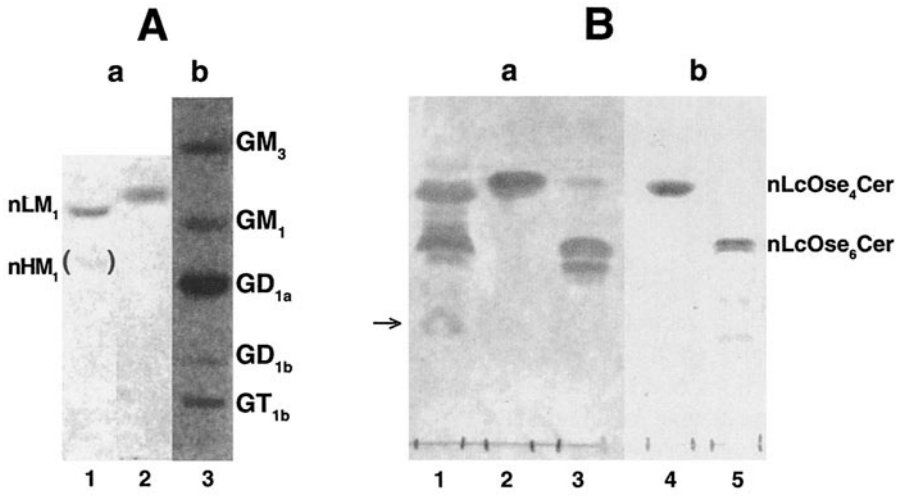

Figure 2. A. HPTLC-immunoblot of nLM1 and nHM1 from embryonic rat cerebral cortex, immunostained with monoclonal antibody NS-24 [18]. Panel a, immunoblot; b, orcinol staining. Lane 1, monosialoganglioside fraction from embryonic day 21 rat cerebral cortex; 2, nLM1 isolated from rat sciatic nerve [21, 22]; 3, ganglioside standards as shown. HPTLC was performed with chloroform:methanol:aqueous 0.25% $CaCl_2$ (5:4:1, by volume) as the solvent. For immunostaining, the HPTLC plate was exposed to antibody NS-24 hybridoma supernate followed by peroxidase-conjugated goat anti-mouse IgM antibodies. The colour was developed with 4-chloronaphthol and H_2O_2 [2]. Antibody NS-24 is more reactive with nLM1 than with the nHM1 band in parenthesis. **B**. HPTLC-immunoblot of nLcOse$_4$Cer and nLcOse$_6$Cer derived from nLM1 and nHM1 by acid hydrolysis, followed by immunostaining with monoclonal antibody 1B2 [20]. HPTLC was performed with chloroform:methanol:aqueous 0.25% $CaCl_2$ (5:4:1, by volume) as the solvent. Panel a, immunoblot; b, orcinol staining. Lane 1, desialylated products of nLM1 and nHM1 in monosialoganglioside fraction from embryonic day 21 rat cerebral cortex; lanes 2 and 4, standard nLcOse$_4$Cer 0.4 μg and 1.2 μg, respectively; lanes 3 and 5, standard nLcOse$_6$Cer 0.125 μg and 0.5 μg, respectively. Doublets are seen in the immunoblot due to higher sensitivity of detection by the antibody as compared to orcinol staining. Arrow shows 1B2 reactive band, possibly a higher neolactosaminyl analogue of nLcOse$_6$Cer.

from rat CNS tissues than that from PNS is attributed to differences in the fatty acid composition of the lipids. Previously, we have shown that nLM1 from rat sciatic nerve contained mostly long chain fatty acids, 22:0, 24:0 and 24:1, which are usually not found in gangliosides from CNS tissues [21]. Similar to nLM1, SGGL-1 from CNS, which contained mainly (> 85%) short chain fatty acids (16:0, 18:0 and 18:1), migrated on HPTLC slightly more slowly than SGGL-1 from PNS which contained a large proportion (~ 60%) of long chain fatty acids (> 18:0) [7].

The developmental expression of nLM1 and nHM1 in the rat cerebral cortex and cerebellum is shown in Fig. 3. The amount of nLM1 was quantitated both by HPTLC-immunostaining with NS-24, and by the release of sialic acid and measuring the reactivity of the product $nLcOse_4Cer$ with 1B2; whereas nHM1 was analysed by the latter method and also by resorcinol reactivity. Similar results were obtained by either of these methods. In the cerebral cortex at embryonic day (ED) 15, the expression of nHM1 was almost three times higher than nLM1 (Fig. 3A). The expression of both these glycolipids declined with development and by postnatal day (PD) 5 the expression was hardly detectable. Beyond PD 5, they were not present in the cerebral cortex.

In the cerebellum, only nLM1 was detected in significant amounts (Fig. 3B). The level of nHM1 in the perinatal and adult cerebellum was very low for quantitative analysis. nLM1 was maximally expressed at PD 15 and its amount declined with age.

The developmental expression of the precursors of these glycolipids, $LcOse_3Cer$, $nLcOse_4Cer$ and $nLcOse_6Cer$ in the cerebral cortex and cerebellum are also given for comparison in the insets of Fig. 3A and B, respectively [14, 15]. The levels of these precursors declined in the cerebral cortex, but increased in the cerebellum with development [14, 15].

Developmental expression of nLD1 and nHD1

Previously we have shown that MAb 7C7, which was raised to ED 15–17 rat forebrain, recognized purified disialogangliosides GD_3 as well as nLD1 and nHD1, but not other disialogangliosides such as GD_{1a} and GD_{1b}, a result indicating the requirement of terminal disialic acid residues for reactivity [2, 6]. The characterization of nLD1 and nHD1 in rat and mouse cerebral cortex and cerebellum was also previously reported by us [6]. The developmental expression of nLD1 and nHD1 in the rat cerebral cortex and cerebellum is shown in Fig. 4. In the cerebral cortex, nLD1 and nHD1 were maximally expressed around ED 19 and they rapidly declined with development (Fig. 4A). By PD 10–15, the expression of both these glycolipids was negligible in the cerebral cortex. In the cerebellum, both nLD1 and nHD1 increased

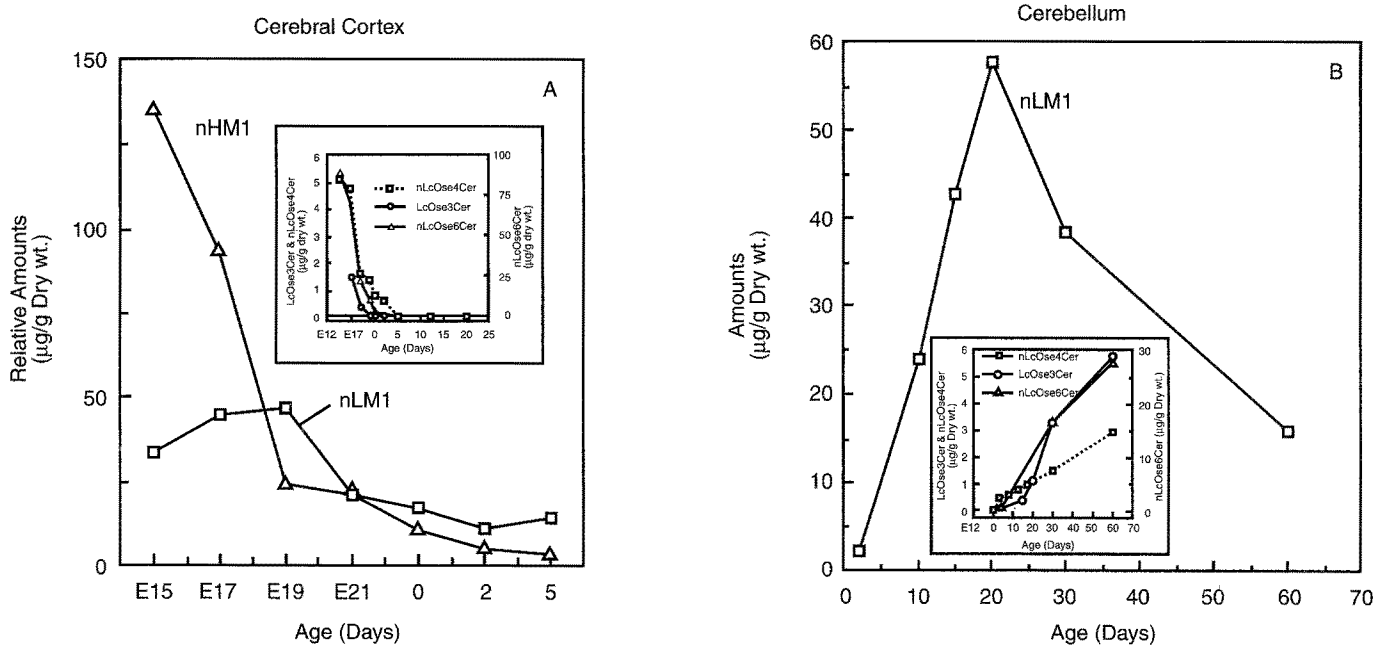

Figure 3. Developmental expression of nLM1 and nHM1 in the rat cerebral cortex (A) and cerebellum (B). The quantitative analysis was as described in the Methods section. Data represent mean values of two-three determinations of each age. The value at each point varied within a range of 9%. The developmental expression of $LcOse_3Cer$, $nLcOse_4Cer$ and $nLcOse_6Cer$ are also given in the insets of the Figures for comparison, from previously published results [14, 15].

with development from around PD 10–15 up to PD 50 (Fig. 4B).

To determine the localization of these two glycolipids in the cerebellum, the lipids were analysed in the microdissected cerebellar layers, obtained from freeze-dried cryocut sections, followed by extraction and HPTLC-immunoblotting with MAb 7C7, as described previously [8]. Gangliosides nLD1 and nHD1 were primarily localized in the Purkinje cell/molecular layer (Fig. 5, lane 3) and in deep cerebellar nuclei (Fig. 5, lane 4) where the Purkinje cell terminate. These gangliosides, however, were not detectable in equivalent amounts of the granule cell layer (lane 5) and white matter (lane 6).

Developmental expression of 9-O-acetyl-nLD1

Previously, we have shown that rodent cerebellum contains several *O*-acetylated gangliosides [6] which were recognized with MAbs ME-311 [23] and 3G5 [16]. We showed that in the disialoganglioside fraction of adult mouse cerebellum, three major *O*-acetyl gangliosides were recognized by MAb 3G5. They were 9-*O*-acetyl-GD3, 9-*O*-acetyl-nLD1 and 9-*O*-acetyl-nHD1 [6]. We also showed that these gangliosides were not detectable in the adult rat cerebral cortex, but 9-*O*-acetyl-GD3, and 9-*O*-acetyl-nLD1 could be identified in the fetal rat cerebral cortex [6]. Here we have studied the developmental expression of 9-*O*-acetyl-nLD1 in the rat cerebral cortex and cerebellum by HPTLC-immunoblotting of the disialoganglioside fraction using MAb 3G5 (Fig. 6). In the rat cerebral cortex, 9-*O*-acetyl-nLD1 was expressed maximally at birth and by PD 10 its level was significantly reduced. In contrast, in the rat cerebellum, it increased almost linearly with age from PD 10 to PD 50.

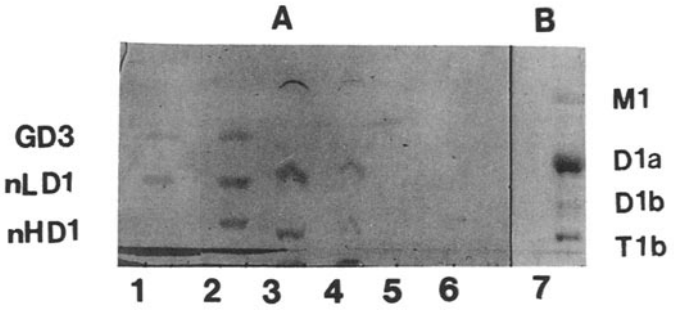

Figure 5. Expression of nLD1 and nHD1 in different cellular layers of adult rat cerebellum. The disialoganglioside fraction, isolated from different cellular layers of the freeze dried cryocut slices of cerebellum [8], was chromatographed on an HPTLC plate and immunostained with MAb 7C7 [6]. Lane 1, standard ganglioside GD3 and nLD1; 2, whole cerebellum; 3, Purkinje cell/molecular layer; 4, deep cerebellar nuclei; 5, granule cell layer; 6, white matter; and 7, standard gangliosides stained with orcinol. In each tissue lane, amounts equivalent to 1 mg of dry weight of tissue were spotted. M1, D1a, D1b and T1b are GM_1, GD_{1a}, GD_{1b} and GT_{1b} respectively.

Developmental expression of SSEA-1/7A reactive fuconeolactoglycolipids

SSEA-1/7A antibody is specific for terminal Galβ1-4GlcNAc–

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1α-Fuc

structure [3]. The Le^x glycolipids in the brain are derivatives of $nLcOse_4Cer$. Two 7A-reactive glycolipids were previously identified in brains of patients with

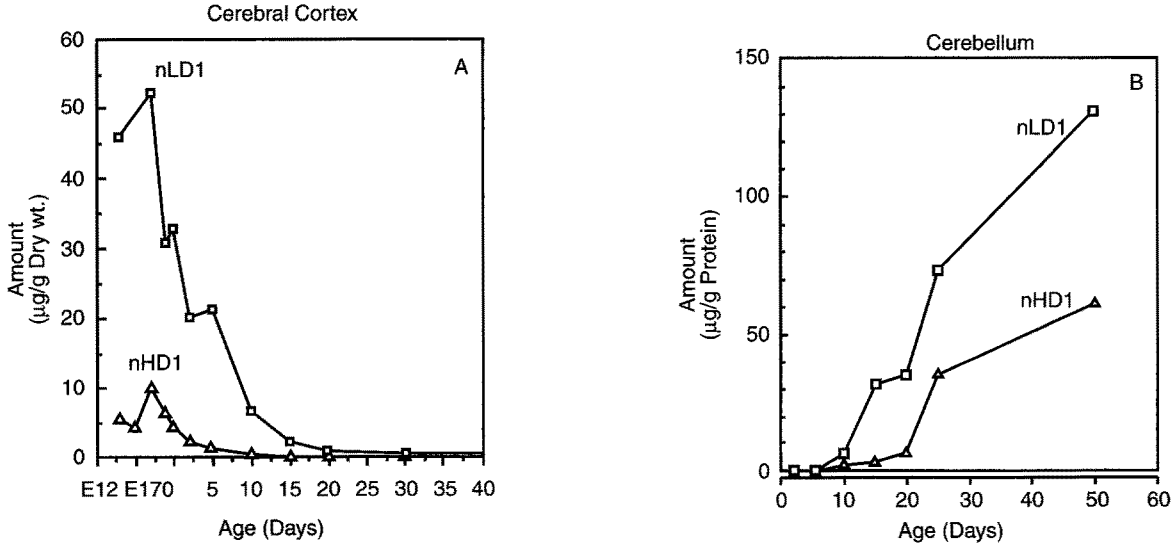

Figure 4. Developmental expression of nLD1 and nHD1 in the rat cerebral cortex (A) and cerebellum (B). The quantitative analysis was as described in the Methods section. Data represent mean values of two–three determinations of each age. The value at each point varied within a range of 12%.

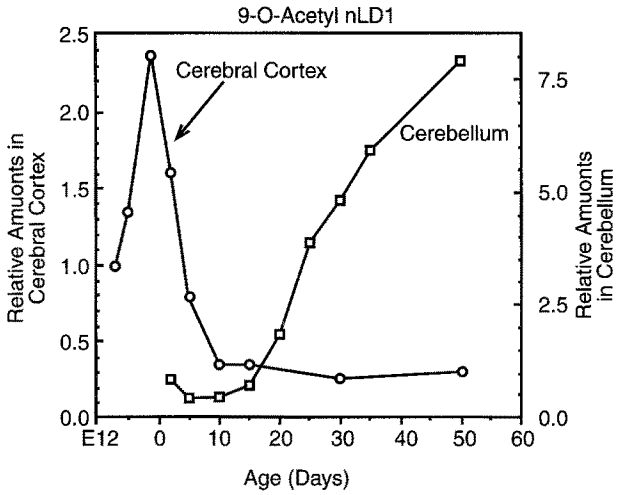

Figure 6. Developmental expression of 9-*O*-acetyl-nLD1 in the rat cerebral cortex and cerebellum. Data represent mean values of two determinations of each age. The value at each point varied within 5–11% range. Since a calibrated standard of 9-*O*-acetyl-nLD1 was not available, relative amounts at each age given are based upon optical density of the immuno-reactive bands on the HPTLC plate.

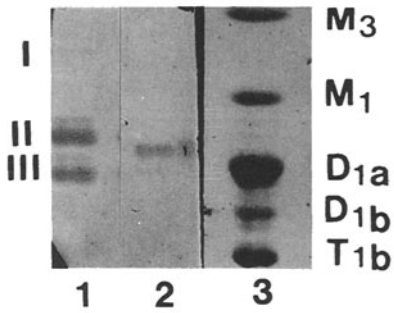

Figure 7. HPTLC-immunoblot of fucosylneolactoglycolipids (Le^x antigens) from embryonic rat cerebral cortex, immunostained with monoclonal antibody 7A [3]. Lane 1, neutral glycolipid fraction from DEAE-Sephadex column; 2, 7A reactive kidney glycolipid [17]; and 3, ganglioside standards stained with orcinol. M_3, M_1, D_{1a}, D_{1b} and T_{1b} are GM_3, GM_1, GD_{1a}, GD_{1b} and GT_{1b}, respectively. Glycolipid I is Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4Glcβ1-1Ceramide; II, Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Gal1-4Glcβ1-1Ceramide; and III, Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4[Fucα1-3]GlcNAcβ1-3Gal1-4Glcβ1-1Ceramide.

fucosidosis [24]. They were presumed to be accumulated in the patients due to lack of their normal catabolism by the enzyme fucosidase [24]. They were characterized as Fucα1-^{3}IIInLcOse$_4$Cer and (Fuc)$_2$α1-^{3}III,^{3}VnLcOse$_6$Cer [24]. In the neutral glycolipid fraction of the normal adult mouse cerebellum we have previously identified the presence of three (I, II and III) 7A-reactive glycolipids [5]. I and III were identified as the above two glycolipids reported in the fucosidosis patient, whereas II was tentatively identified as Fucα1-^{3}V-nLcOse$_6$Cer, based on the relative mobility with the other two [5] and a 7A-reactive kidney glycolipid with a globo-core structure:

Galβ1–4GlcNAcβ1–6GalNAcβ1–3Galα1–4Galβ1–4Glcβ1–1 ceramide [17].
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α1Fuc β1Gal

The presence of MAb 7A reactive glycolipids (I-III) in the embryonic rat cerebral cortex is shown in Fig. 7. The developmental expression of these glycolipids in the rat cerebral cortex is shown in Fig. 8A. The relative amounts of the glycolipids determined are based upon reactivity with 7A antibody on HPTLC plates. It has been previously noticed that 7A does not react as well with Fucα1-^{3}IIInLcOse$_4$Cer as with the other two glycolipids, even though equivalent amounts of the three glycolipids, based upon orcinol reactivity, were tested [5, 17]. Therefore, the actual amount of Fucα1-^{3}IIInLcOse$_4$Cer may be more than shown here. (Fuc)$_2$-nLcOse$_6$Cer and Fuc-nLcOse$_4$Cer were maximally expressed in the cerebral cortex at ED 17 and then rapidly declined and by PD 5–10 they were almost undetectable. The amount of Fuc-nLcOse$_6$Cer was maximum at E15 and declined steadily thereafter similar to (Fuc)$_2$-nLcOse$_6$Cer.

In the rat cerebellum, Fuc-nLcOse$_4$Cer was barely detectable and could not be quantitated reliably. The level of Fuc-nLcOse$_6$Cer in cerebellum, in contrast to that in the cerebral cortex, was also very low (Fig. 8B). (Fuc)$_2$-nLcOse$_6$Cer was the major 7A-reactive glycolipid in the cerebellum. Its expression was maximal at around PD 7, and declined with development. It appears that 7A-reactive glycolipids and nLM1 are the only neolactoglycolipids which decline with development of the cerebellum, in contrast to other neolactoglycolipids so far studied.

Discussion

A number of lacto and neolacto series of glycolipids have been isolated and characterized mainly as blood group antigens A, B, H, Lewis, Ii and P1 [25]. These glycolipids and their sialosyl derivatives also constitute the major glycolipid components of various tissues and organs [25]. Until recently, lacto and neolacto series glycolipids have been considered to be absent in brain [25], although the presence of nLM1 in human and rat peripheral nerves has been known [21, 26]. Vanier *et al.* [27] first reported a small quantity of lacto-*N*-fucopentaosyl (III) ceramide and its sialosyl derivative in brain. Later 3-sulfoglucuronyl neolactoglycolipids, SGGLs were characterized in the embryonic central nervous system [2, 7]. It was shown that SGGLs were expressed maximally only in the embryonic

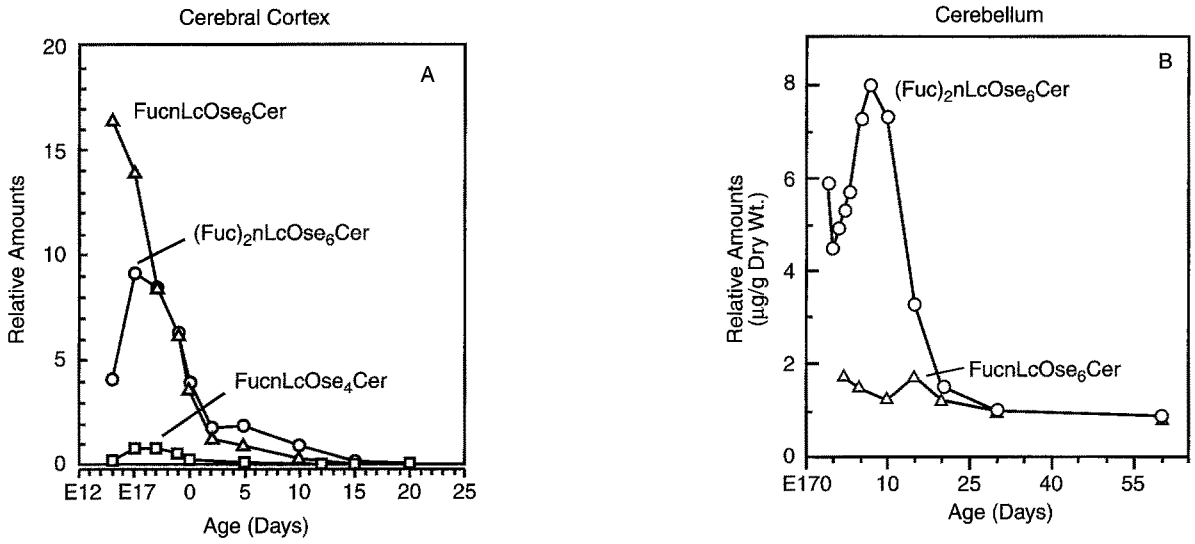

Figure 8. Developmental expression of monoclonal antibody 7A reactive fucosylneolactoglycolipids (Le^x antigens) in the rat cerebral cortex (A) and cerebellum (B). Data represent mean values of two determinations at each age. In panel A, relative amounts at each age are based upon optical density of the immuno-reactive bands on the HPTLC plate. In panel B, the relative amounts at each age are based upon comparison of the immuno-reactive bands with the known amounts of the 7A reactive kidney glycolipid standard [17].

rodent cerebral cortex and disappeared in the postnatal animals, whereas they were expressed biphasically in the cerebellum [2, 7, 8]. It was shown that lactosylceramide:GlcNAc-transferase (GlcNAc-Tr), involved in the synthesis of $LcOse_3Cer$, was the key regulatory enzyme controlling the differential expression of SGGLs. This was also confirmed by analysis of the availability of the endogenous precursors, viz $LcOse_3Cer$, $nLcOse_4Cer$ and $nLcOse_6Cer$ [7, 8, 14, 15] (see insets Fig. 3). The activity of GlcNAc-Tr and the levels of these precursors declined in the cerebral cortex and were no longer detectable by PD 5, whereas in the cerebellum they increased with development (reviewed in [10]). $LcOse_3Cer$ is the key early intermediate in the biosynthesis of all lacto and neolactoglycolipids and the availability and synthesis of this precursor should regulate the expression of other lacto and neolactoglycolipids, besides SGGLs. Our results presented here show that in fact all neolactoglycolipids identified in the embryonic cerebral cortex, such as nLM1, nHM1, nLD1, nHD1, 9-OAc-nLD1, Fuc-$nLcOse_4$-Cer, Fuc-$nLcOse_6Cer$ and $(Fuc)_2$-$nLcOse_6Cer$ are down regulated in the postnatal animals and are lost in the adult, similar to SGGLs. On the other hand, in the cerebellum most of these glycolipids increase with development due to increasing levels of $LcOse_3Cer$ and $nLcOse_4Cer$. nLM1 and 7A reactive fuconeolactoglycolipids are the only neolactoglycolipids which declined in the maturing cerebellum, starting at around PD 10–15. It is likely that these lipids could be regulated by the activity of other glycosyltransferases, or by their rapid turnover due to changes in the activity of the degradative enzymes, with development.

nLM1 and nHM1

The presence of nLM1 and 3′-isoLM1 (IV^3NeuAc-$LcOse_4Cer$) has been reported in the fetal human brain [9, 28]. Here we report the presence of nLM1 in rat cerebral cortex and cerebellum for the first time. The presence of nHM1 has not been reported previously in the central nervous system. However, we have reported its presence in the myelin membranes of rat sciatic nerve [21, 22]. nHM1 is present at much higher levels than nLM1 in the embryonic cerebral cortex (Fig. 3A). In the cerebellum, the level nHM1 is very low.

In the rat cerebral cortex, the rates of decline of nLM1 and nHM1 were very similar to their direct precursors, $LcOse_3Cer$, $nLcOse_4Cer$ and $nLcOse_6Cer$ (Fig. 3A inset). This indicates that the availability of these precursors most likely regulates the expression of nLM1 and nHM1 in the cerebral cortex. On the other hand, in the cerebellum, nLM1 was maximally expressed at PD 15 and then declined, even though the availability of the precursor $nLcOse_4Cer$ continued to increase up to PD 60 (Fig. 3B, inset). This would suggest that in the cerebellum besides availability of $nLcOse_4Cer$, nLM1 may also be regulated by: (a) the down regulation of the enzyme $nLcOse_4Cer$:2-3 sialyltransferase or (b) by the up regulation of nLM1:2-8 sialyltransferase for the synthesis of nLD1 and 9-O-Ac-nLD1 or (c) by the up regulation of the activity of the degradative enzyme sialidase. The

expression of nLD1 in the cerebellum sharply increased by PD 15 (Fig. 4B). This would suggest that beyond PD 15 the down regulation of nLM1 could be due to its conversion to nLD1. Studies on the activities of the enzymes involved in the metabolism of these glycolipids in the cerebral cortex and cerebellum would resolve how these glycolipids are regulated.

nLD1 and nHD1

Previously we have reported the presence of nLD1 and its higher analogue nHD1 in the adult cerebellum and the embryonic cerebral cortex [6] and have completely characterized the structure of nLD1 [6]. nLD1 is one of the major gangliosides of rodent cerebellum [6]. During development of the cerebral cortex, the expression of nLD1 and nHD1 is maximal around ED 19 and both these gangliosides are down regulated with age. The rate of disappearance of nLD1 and nHD1 from the cerebral cortex is in accordance with the down regulation of their precursors LcOse3Cer, nLcOse4Cer and nLM1, as well as nLcOse$_6$Cer and nHM1, respectively (see Fig. 3A and 4A). In the cerebellum, both nLD1 and nHD1 increase with development according to the availability of their respective precursors (Fig. 3B and 4B).

9-O-Acetyl-nLD1

Recently, 9-*O*-acetylated gangliosides, 9-*O*-Ac-GD3 and 9-*O*-Ac-nLD1 which react with MAbs such as, 3G5 [16], ME 311 [23], D1.1 [29], Jones [30], P-path [31] and GMR [32] have attracted much attention due to their specific pattern of topographical expression in the neural and other tissues as determined by immunocytochemistry [29–35]. These gangliosides have been implicated in cell differentiation, migration and pattern formation. Previously, we have shown that in the adult rodent cerebellum the major 9-*O*-acetyl disialosyl-ganglioside was 9-*O*-Ac-nLD1 [6]. Here we have shown that this ganglioside increased in the rat cerebellum almost linearly from PD 10 similar to its immediate precursor nLD1; whereas in the rat cerebral cortex it was down regulated from birth onwards, similar to nLD1 in that tissue. Several studies [31, 32, 36] have confirmed our initial findings that 9-*O*-Ac-nLD1 is specifically localized in Purkinje cells and their dendrites in the cerebellum [6]. The specific cellular localization of this ganglioside in the cerebral cortex remains to be investigated.

Fucosyl-neolactoglycolipids

The SSEA-1/7A reactive Le^x antigen is highly regulated during development and has been implicated in cellular interactions [37]. Le^x epitope is expressed on glycolipids, glycoproteins and proteoglycans and has been shown to be developmentally regulated in the nervous system [3, 38, 39]. Le^x epitope is recognized by several slightly different but related antibodies such as SSEA-1 [40], MMA/CD-15 [38], 7A [3], 43B11 [41] and Forse I [42]. These antibodies have specificity not only towards the terminal Galβ1-4 [Fucα1-3]GlcNAc-structure, but also appear to have relative fine specificity depending upon the location of the terminal carbohydrate epitope; i.e. whether it is on lipids or proteins as well as on the length and type of the additional carbohydrate chain linking the terminal epitope. The use of different antibodies for the immunocytochemical detection of the Le^x epitope by different investigators has generated variable results in similar systems [3, 38, 39]. Yamamoto *et al.* [3], using the more lipid reactive MAb 7A, as used here, have shown that in the ED 13–15 rodent cerebral cortex, Le^x staining was confined to rapidly dividing cells in the ventricular and subventricular zone. The expression of Le^x antigens disappeared postnatally when cell division and cell migration was nearly complete [3]. Our biochemical results in this report have shown that the three 7A-reactive glycolipids are differentially expressed during embryonic development and are rapidly down regulated after birth in accordance with the observations by immunostaining of the cerebral cortex [3]. The down regulation is most likely due to the loss of the enzyme GlcNAc-Tr which synthesizes LcOse3Cer, the key precursor of these glycolipids. Our immunocytochemical studies in the adult rat cerebellum with MAb 7A failed to identify any reactivity with specific cellular structures [unpublished]. The latter observation is in accordance with the biochemical loss of these glycolipids beyond PD 25, observed in the cerebellum (Fig. 8B).

In the cerebellum, the two fuco-neolactoglycolipids, unlike the other neolactoglycolipids, were down regulated by PD 20, despite the increasing levels of their direct precursor nLcOse6Cer (see inset Fig. 3B). This would indicate that the expression of these glycolipids in the cerebellum is regulated by factors other than the availability of the precursor, such as the activity of the enzyme fucosyl transferase. Experiments are in progress to determine the activity of this enzyme during the development of the cerebellum.

Cellular localization of neolactoglycolipids in cerebellum

Previously, we have shown that the entire neolacto series of glycolipids are significantly reduced in the cerebellum of murine mutants, such as Purkinje cell degeneration (pcd/pcd), staggerer (sg/sg), lurcher (Lc/+), nervous (nr/nr) and leaner (tg^{la}/tg^{la}) in which Purkinje cells are abnormal [4, 5, 6, 43]. These glycolipids are nearly normal in other cerebellar mutants such as quaking (qk/qk), weaver (wv/wv) and reeler (rl/rl) where Purkinje cells are not primarily involved. We have also shown that the enzyme GlcNAc-Tr which synthesizes LcOse$_3$Cer is completely absent in the cerebellum of pcd/pcd and sg/sg mutants in which Purkinje cells were severely lost [44]. These results show that the neolactoglycolipids in the adult cerebellum

are mainly associated with Purkinje cells and their related structures such as dendrites and axon terminals, but not with other cell types. Biochemical analyses and immunocytochemical experiments have confirmed that neolactoglycolipids such as SGGLs are mainly associated with Purkinje cells [8, 43, 45].

Although cellular and subcellular fine localizations appear convincing by immunocytochemical methods, these methods have severe limitations, especially when the same epitope is expressed on multiple antigens, such as on several glycolipids, glycoproteins and proteoglycans, leading to confusing results. Therefore, biochemical analyses are important in corroborating immunocytochemical observations. Reynolds and Wilkin [46], using an immunocytochemical method, reported that GD3 was localized on Purkinje cells, whereas Kotani *et al.* [32] found that GD3 was expressed on granule cells and white matter. These conflicting results may be attributed to the fact that the antibodies used by these investigations not only react with the terminal diNeuAc group of GD3, but also with that of LD1 and HD1. In addition, different tissue fixation techniques used in different laboratories may also cause variable results. Our biochemical analyses of nLD1 and nHD1 in the different cerebellar layer presented here (Fig. 6) clearly show that these glycolipids are associated with Purkinje cells and their dendrites in the molecular layer and their axon terminals in the deep cerebellar nuclei where Purkinje cell terminate, but are absent in the granule cell layer and white matter. The precise function of the neolactoglycolipids associated with the Purkinje cells remains to be determined.

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