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Source: Zoological Science, 15(1) : 27-34

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.15.27

ZOOLOGICAL SCIENCE **15**: 27–34 (1998)

Compositional Changes in Glycoconjugates Recognized Histochemically with Lectins in Purkinje Cells in Suckling and Adult Rats

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ABSTRACT—Cerebella from suckling and adult rats were examined histochemically with 19 different biotin-labeled lectins. Purkinje cells from postnatal rats had a marked ability to combine with many lectins, but minimal ability was found in adult rats except for Con-A, LEL, and MAL lectins. The cell body of Purkinje cells on postnatal day 7 was strongly labeled with 6 lectins (Con-A, LTL, MAL, SJA, UEA-I, and VVA). Only moderate staining was observed with these lectins on postnatal day 5. The dendritic tree of the cells showed a moderate labeling ability with LTL and UEA-I on postnatal days 15 and 20. The dendritic tree was strongly labeled with MAL on postnatal days 10, 15, 20 and adult. Positive reactions were observed in the cells when cerebellar sections from rats on postnatal day 7 were incubated with 3 other lectins (AAL, LEL, and SBA). The cells on postnatal day 7 were rarely labeled with BSL-II, DBA, DSL, LCA, PNA, PSA, RCA120, SSA, STL, and WGA. Purkinje cells on postnatal day 7 may be rich in N-linked oligosaccharides, with terminal sugar structures that resemble blood-group-related antigens (type H) and/or tumor-related antigens. These glycoconjugates may be present at low levels in the Purkinje cells of adult rats. Dendrites of Purkinje cells of adult rats were strongly labeled by Con-A, LEL, and MAL. The dendrites of Purkinje cells may be rich in highly branched oligosaccharides.

INTRODUCTION

The cerebellum of rodents develops rapidly during the suckling period (Altman, 1972), and intercellular interactions during this period are essential for normal cerebellar development (Changeux and Mikoshiba, 1978). It is known that intercellular communication during cerebellar development is mediated by many glycoproteins. The migration of cerebellar granule cells from the external granule layer to the internal granule layer is mediated by glycoproteins such as N-CAM, cytotactin, and L1 (e.g., Choung *et al*., 1987). The migration of Purkinje cells is also guided on radial fibers by tenascin (Yuasa and Kawamura, 1992). The functions of core proteins of these glycoproteins have been analyzed in detail (e.g., Oohira *et al*., 1994), but relatively little attention has been paid to the developmental changes in glycans. Specific types of glycan appear in the central nervous system during development. Zanetta *et al*. (1978) demonstrated the emergence of glycoprotein and lectin-like molecules on growing parallel fibers and Purkinje cells, respectively. They hypothesized that both lectin-like molecules and glycans may be recognition molecules that allow specific contacts between parallel fibers and Purkinje cells during synaptogenesis (Zanetta *et al*., 1985a, b). More recently, it was reported that a membrane-bound heparan sulfate proteoglycan were transiently expressed on newly formed parallel fibers during the suckling period (Watanabe *et al*., 1996). In this study, we examined histochemically the ability of 19 kinds of lectins to combine with Purkinje cells in the cerebellum, with emphasis on the transient accumulation of glycans in Purkinje cells during the suckling period.

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MATERIALS AND METHODS

Rats of the Sprague-Dawley strain during the suckling period (postnatal days 0, 5, 7, 10, 15 and 20) and in adulthood (4 to 6 months old) were used in this study. Prior to sacrifice and analysis, the rats were deeply anesthetized with chloroform. The cerebellum was removed, and the vermis was sectioned saggitally into 2-mm-thick slices. The slices were immersed in Carnoy's solution for 3 hr and subjected to serial paraffin sectioning (5-μm thick). Some cerebella were subjected to frontal paraffin sectioning. 19 different biotinylated lectins were used in this study (Table 1). 17 biotinylated lectins (BSL-II, Con A, DBA, DSL, LCA, LEL, MAL, PNA, PSA, RCA120, SBA, SJA, SSA, STL, UEA-I, VVA and WGA) were purchased from Funakoshi Co. LTD (Tokyo, Japan) and 2 biotinylated lectins (AAL and LTL) from Seikagaku-Kogyo Co. LTD (Tokyo, Japan). Samples were incubated

in a solution containing 50 to 2500 ng ml^{-1} of biotinylated lectins in 125 μl of buffer-A per section (for 120 min at 25°C). Buffer-A contained 50 mM Tris-HCl (pH 7.4), 0.5 M NaCl, 0.05% Tween 20, and 3% bovine serum albumin. The location of lectins was visualized by the avidin-biotin-peroxidase method with diaminobenzidine (Dojindo Co. Ltd. Kumamoto, Japan) as the chromogen. The optimum concentration of lectins giving the strongest positive signal were chosen by serial dilution.

The extent of staining of Purkinje cells by each lectin was graded according to an arbitrary scale of (–) to (++), as follows: (–), Purkinje cells showed no or very weak ability to combine with the lectin under conditions whereby other cerebellar structures were strongly stained; (+), the lectin strongly stained cerebellar structures, but Purkinje cells were stained to only a limited extent; (++), Purkinje cells were clearly stained by the lectin and the cells were easily distinguishable from other cerebellar structures.

To validate the specificity of the lectin-combining reactions, we performed enzyme-digestion control and monosaccharide-blocking control experiments, as described below. Sections were incubated at 37°C for 120 min in a solution containing α-L-fucosidase (2.5 μU/section) in 0.1 M citrate buffer, pH 4.5, and then were incubated with biotinylated lectins (UEA-I or LTL). Controls for the lectin labeling were run by incubating the sections in biotin-conjugated lectins with the addition of hapten sugars (10 to 40 mM L-fucose/ LTL and UEA-I; 10 mM methyl-α-D-mannopyranoside/Con A).

RESULTS

Results of the histochemical analysis of Purkinje cells with the various lectins are summarized in Table 2. The staining ability of Purkinje cells for various biotinylated lectins markedly changed with development. Changes in the staining for lectins could be categorized into five groups: (A), staining was markedly positive during the neonatal stage but became nega-

Table 1. Lectins used in this study and their major specificities

Acronym (Source)	Major specificity	References
LTL (*Lotus tetragonolobus*)	4GLβ1-4(L-Fα1-3)GLβ1-	Kirkeby *et al.*, 1993 Whyte *et al.*, 1993
SJA (*Sophora japonica*)	Gα1-3Gβ1-4GL	Pretz, 1983
UEA-I (*Ulex europaeus*)	Fα1-2Gβ1-4GL	Osawa and Matsumoto, 1983 Kirkeby *et al.*, 1993
VVA (*Vicia villosa*)	α or β-GA	Jones *et al.*, 1993
LEL (*Lycopersicon esculentum*)	(GL)n, n = 3 or 4	Hentschel and Walther, 1993
MAL (*Maackia amurensis*)	Siaα2-3Gβ1-4GL	Wang and Cummings, 1988
Con A (*Canavalia ensiformis*)	Mα1-6 (Mα1-3) M	Kornfeld and Ferris, 1975 Agrawal and Goldstein, 1983
AAL (*Aleuria aurantia*)	Fα1-6GL > Fα1-GL	Jones *et al.*, 1993 Kirkeby *et al.*, 1993
SBA (*Glycine max*)	GAα1-3G	Lis and Sharon, 1978 Lis and Sharon, 1983
BSL-II (*Bandeiraea simplicifolia*)	α- or β-GL	Jones *et al.*, 1993
DBA (*Dolichos biflorus*)	GAα1-3GA	Baker *et al.*, 1983 Jones *et al.*, 1993
DSL (*Datura stramonium*)	G1-4GL1-2(Gβ1-4GL1-4) M	Baker *et al.*, 1983 Crowley *et al.*, 1984
LCA (*Lens culinaris*)	Fα1-6GL, α-D-M, α-D-GL	Sage and Green, 1983
PNA (*Arachis hypogaea*)	Gβ1-3GA	Lotan *et al.*, 1975
PSA (*Pisum sativum*)	α-D-M, α-D-GL, Fα1-6GL	Cummings and Kornfeld, 1982
RCA120 (*Ricinus communis*)	(Gβ1-4GL)n, n = 3 or 4	Baenziger and Fiete, 1979
SSA (*Sambucus sieboldiana*)	Siaα2-6G	Shibuya *et al.*, 1989
STL (*Solanum tuberosum*)	(GL)n, n = 3 or 4	Whyte *et al.*, 1993
WGA (*Triticum vulgaris*)	GLβ1-4Mβ1-4GLβ1-4GL	Marchesi, 1983

F, Fucose; G, Galactose; M, Mannose; GA, N-acetyl galactosamine; GL, N-acetyl glucosamine.

Table 2. Stainability of developing Purkinje cells with biotinylated lectins

Lectin	postnatal day						Adult
	0	5	7	10	15	20	
LTL	+	++	++	++	+	+	+
SJA	+	+	++	++	+	+	−
UEA-I	+	++	++	++	+	+	−
VVA	+	+	++	+	+	+	+
LEL	+	+	+	+	+	++	++
MAL	+	+	++	++	++	++	++
Con A	+	++	++	++	++	++	++
AAL	+	+	+	+	+	+	+
SBA	−	−	+	+	+	+	+
BSL-II	−	−	−	−	−	+	+
DBA	−	+	+	+	−	−	−
DSL	−	−	+	−	−	−	−
LCA	−	−	−	−	−	+	+
PNA	−	−	−	−	−	−	−
PSA	−	−	−	−	−	−	−
RCA120	−	−	−	−	−	−	−
SSA	−	−	−	−	−	−	−
STL	−	−	−	−	−	−	−
WGA	−	−	−	−	−	+	+

See text for explanation of symbols.

tive with development; (B), staining was weak in the neonatal stage but became strongly positive with development; (C), staining of the cells was consistently and markedly positive; (D), staining was weak but consistently positive; and (E), staining was consistently negative.

LTL, SJA, UEA-I and VVA were categorized as group A. Purkinje cells were slightly stained with these lectins on postnatal day 0. The lectins markedly stained the cells (UEA-I; Fig. 1A) on postnatal day 5. The edge of somata and granular structures in the apical cone of the cells were clearly stained with the lectins. The lectin reactions appeared slightly on the dendrite of the cells. On postnatal day 7, a strong reaction of the lectins appeared on the cell surface and granular structure in the somata and dendrite (LTL; Fig. 2A, SJA, Fig. 2B; VVA, Fig. 2C). In particular, somata and dendrites of the cells were heavily stained with UEA-I (Fig. 1B). The cell surface and granular structures in the dendrites of postnatal day 10 rats were strongly stained with LTL, SJA and UEA-I (Fig. 1C). The cells were slightly stained with VVA on postnatal day 10. The dendrite of Purkinje cells on postnatal days 15 and 20 were sharply defined with LTL (data not shown) and UEA-I (Fig. 1D and E, respectively), but the somata was slightly stained with the lectins. The somata and dendrites of the cells were slightly stained with SJA and VVA on postnatal days 15 and 20. No or very slight reactions with the cells were observed when cerebellar sections from adult rats were incubated with LTL(Fig. 2D), SJA (Fig. 2E), UEA-I (Fig. 1F) or VVA(Fig. 2F). The glomerulus of adult rats was stained with LTL and VVA. White matter of adult rats was strongly stained with LTL, SJA, UEA-I and VVA.

LEL and MAL were categorized as group B. The staining was weak in the neonatal stage but became strongly positive with development. The lectins faintly stained the Purkinje cells on postnatal day 0. MAL markedly stained the cells (Fig. 3A) on postnatal day 5. Granular structures in the apical cone of the cells were clearly stained with the lectin. On postnatal day 7, a strong reaction of the lectin appeared on the somata and dendrites (Fig. 3B). Granular structures on the surface of the somata and dendrites were markedly positive (arrowhead). Some granules in the somata were stained with MAL on postnatal days 10, 15, 20 and adulthood. The dendrites of the Purkinje cells were intensely labeled with MAL (Fig. 3). MAL markedly stained the granular structures on the surface of the dendrites (arrowhead). LEL stained some granules in the somata of Purkinje cells. The reaction was weak on postnatal days 7, 10 and 15. LEL positively stained the granules on postnatal day 20 and adult rats. The molecular layer and glomerulus of adult rats were stained with LEL.

Con-A was categorized as group C. Some granules in the Purkinje cell somata of postnatal day 0 rats were stained with Con-A. The granules in the somata and dendrite were markedly stained on postnatal day 5, 7 (Fig. 4A), 10, 15 and 20. The granules in the somata and fine granules in the dendrite of adult rats (Fig. 4D) were also stained with Con-A. The molecular layer and glomerulus of adult rats were stained with Con-A.

Group D consisted of AAL and SBA. The lectins weakly bound with granules in the Purkinje cell somata at any age. The molecular layer and glomerulus of adult rat cerebella were also weakly stained with the lectins.

Group E consisted of 10 lectins (LCA, BSL-II, DBA, DSL, PNA, PSA, RCA120, SSA, STL, WGA). No or only very slight reactions with the Purkinje cells were observed when cerebellar sections were incubated with the lectins. Although Purkinje cells were rarely labeled with these lectins, some other cerebellar structures were strongly stained with the lectins. Endothelial cells of cerebellar blood vessels were heavily stained with BSL-II, DSL (Fig. 4B), LCA, STL, and WGA at all developmental periods. Bergmann fibers of suckling rats were stained with DSL, STL and WGA. The fibers on postnatal days 7, 10 and 15 were markedly stained with DSL (Fig. 4B). After 20 days of life, no Bergmann fibers were stained with the lectins (Fig. 4E). Some granular structures in the external granular layer were strongly stained with PNA (Fig. 4C). Parallel fibers in the molecular layer were stained with PNA. The intensity of PNA staining on the molecular layer gradually increased with rat development. The molecular layer of adult rat cerebella was markedly stained with PNA (Fig. 4F).

The molecular layer of adult rats was also stained with AAL, BSL-II, Con A (Fig. 4D), DSL (Fig. 4E), LEL, PNA (Fig. 4F), VVA, and WGA. The glomerulus of adult rats was stained with AAL, BSL-II, Con A, DSL, LCA, LEL, LTL, PNA, SSA, VVA, and WGA. White matter of adult rats was strongly stained with BSL-II, DBA, LEL, LTL, SJA, SBA, UEA-I, and VVA.

No or very slight reaction for Con-A was observed on Purkinje cells of postnatal day 7 rats when cerebellar sections

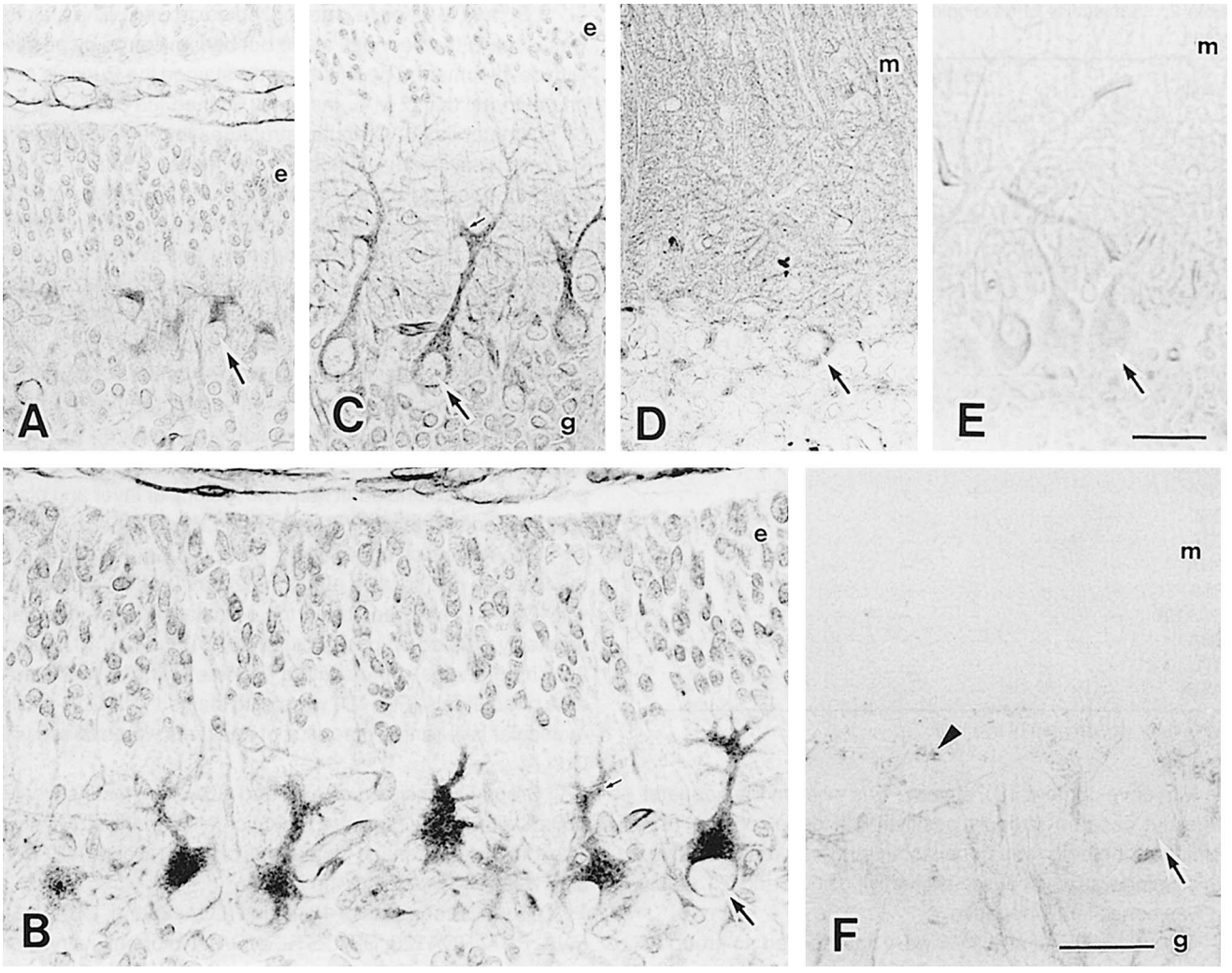

Fig. 1. Photomicrographs of rat cerebella stained with UEA-I. The soma of Purkinje cells (arrow) were strongly labeled with UEA-I on postnatal day 7 (**B**) and moderately labeled on postnatal days 5 (**A**) and 10 (**C**). The dendrites of Purkinje cells reacted with UEA-I on postnatal days 7 (**B**), 10 (**C**), 15 (**D**), and 20 (**E**). Granular structure on the dendrites was stained strongly (small arrow). No reaction with UEA-I was observed in Purkinje cells of adult rat cerebella (**F**). Granular structures in Bergmann-glial cells (arrowhead) were stained with UEA-I in adult rats. Abbreviations: e, external granule cell layer; m, molecular layer; g, granule cell layer. Bar, 30 μm.

were subjected to the staining reaction in the presence of 2 mM methyl-α-D-mannopyranoside. The cells were not or faintly stained with LTL or UEA-I in the presence of α-L-fucose at a concentration of 10 and 40 mM, respectively. The cells were faintly stained with LTL, when sections were incubated with 2.5 μU of α-L-fucosidase for 120 min at 37°C followed by staining with LTL (data not shown).

DISCUSSION

Con-A (Group C) stained the Purkinje cells consistently positive at all ages. Purkinje cells of rat cerebellum may be rich in N-linked oligosaccharides in all developmental stages because the carbohydrate domain common to Con A is the branched chain of an N-linked oligosaccharide with two GlcNAcβ1-2Manα1 non reducing termini linked to a mannose residue at the core (Kornfeld and Ferris, 1975; Agrawal and Goldstein, 1983). Indeed, the reaction with Con A was effectively inhibited by methyl-α-D-mannopyranoside (10 mM). Granular structures in somata and dendrites of the Purkinje cells were strongly stained with Con-A at any age. The granules may be Golgi and/or endoplasmic reticulum membrane because it is well known that the structures in Purkinje cells of adult rats are rich in Con-A binding sites (Wood *et al*., 1974). The localization of the reaction products for various biotinylated lectins in Purkinje cells of suckling rat cerebellum markedly changed with development. The terminal structure of the oligosaccharide may change with development. The Purkinje cells of suckling rat may be rich in oligosaccharide with fucose residue because UEA-I and LTL (group A lectins) combined strongly with Purkinje cells in the cerebella of suckling rats, especially on postnatal day 7. This idea is also supported by the findings of UEA-I (Osawa and Matsumoto, 1983) and LTL (Kirkeby *et al*., 1993; Pereira and Kabat, 1974) having

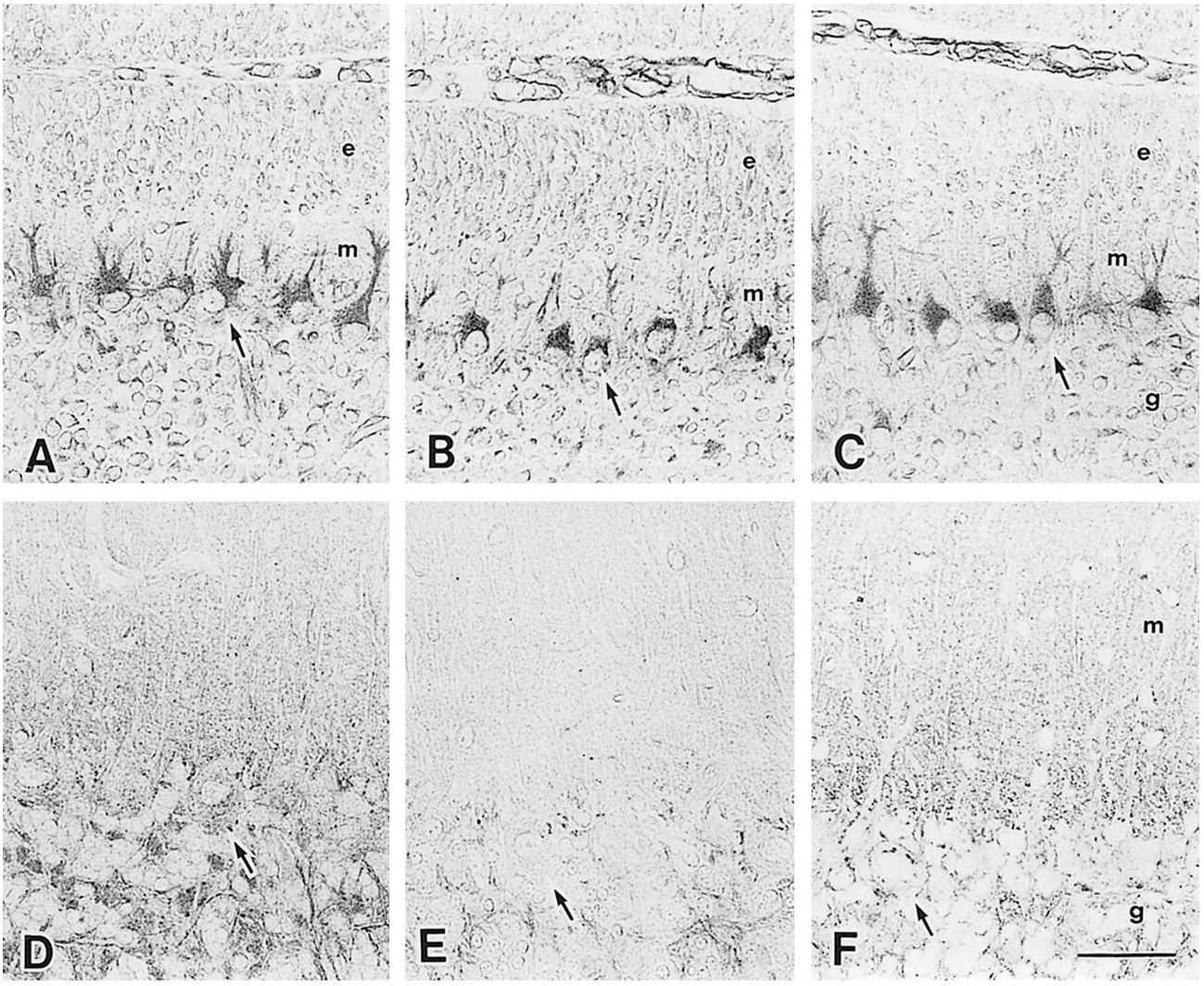

Fig. 2. Lectin histochemical demonstration of glycans in the cerebella of postnatal day 7 and adult rats. **A**, **B** and **C** show cerebella of postnatal day 7 rat. **D**, **E** and **F** were cerebella of adult rats. **A** and **D** were stained with LTL. **B** and **E** were stained with SJA. **C** and **F** were stained with VVA. Purkinje cells (arrow) of postnatal day 7 rats were strongly stained with these three lectins. The Purkinje cells of adult rats were no or slightly stained with the lectins. Abbreviations: e, external granule cell layer; m, molecular layer; g, granule cell layer. Bar, 30 μm.

high affinity for terminal fucose-sugar groups (Fucα1-2Gal1-3GlcNAc), and the finding of the labeling with UEA-I and LTL becoming weak with the presence of L-fucose (30 mM) in the incubation medium for staining. Additionally, the cells in the sections treated with α-L-fucosidase showed a weak ability to combine with LTL. LTL and UEA-I strongly combined with Purkinje cells, but the other three lectins which had affinity for the fucose residue combined weakly. AAL combined moderately with the cells of neonatal rat cerebellum (group D). LCA and PSA scarcely combined with the cells at any developmental stage (group E). AAL (Jones *et al*., 1993), LCA (Sage and Green, 1983) and PSA (Cummings and Kornfeld, 1982) have affinity for sugars with the structure Fucα1-6GlcNAcβ1-4Asn, while UEA-I and LTL have very weak affinity for such structures (Kirkeby *et al*., 1993). The Purkinje cells of suckling rat may be rich in Fucα1-2Gal1-3GlcNAc, but poor in Fucα1-6GlcNAcβ residues. We will temporarily designate this glycan FGP7 (*F*ucosyl-*G*lycan in *P*urkinje cell on postnatal day *7*) in this report. The terminal sugar structure of FGP7 resembled with blood-group-related antigens, type H and Lewis Y. It is quite possible that FGP7 is closely related to developmental processes associated with Purkinje cells because such blood-group-related antigens have been found in embryonic and tumor cells, and various functions for them in development have been postulated (Chandrasekaran *et al*., 1983; Pour *et al*., 1988). The Purkinje cells of postnatal day 7 rat did not react with anti-Lewis Y anti-serum (data not shown).

While Purkinje cells accumulated large amounts of FGP7 on postnatal day 7, this accumulation was only transient. LTL and UEA-I strongly combined with small granules in the somata of the cells on postnatal days 5, 7 and 10, but they combined scarcely to the granules of the cells from rats which were more than 15 days of age. LTL and UEA-I combined to dendrites of the cells on postnatal days 10, 15 and 20 (Fig. 2).

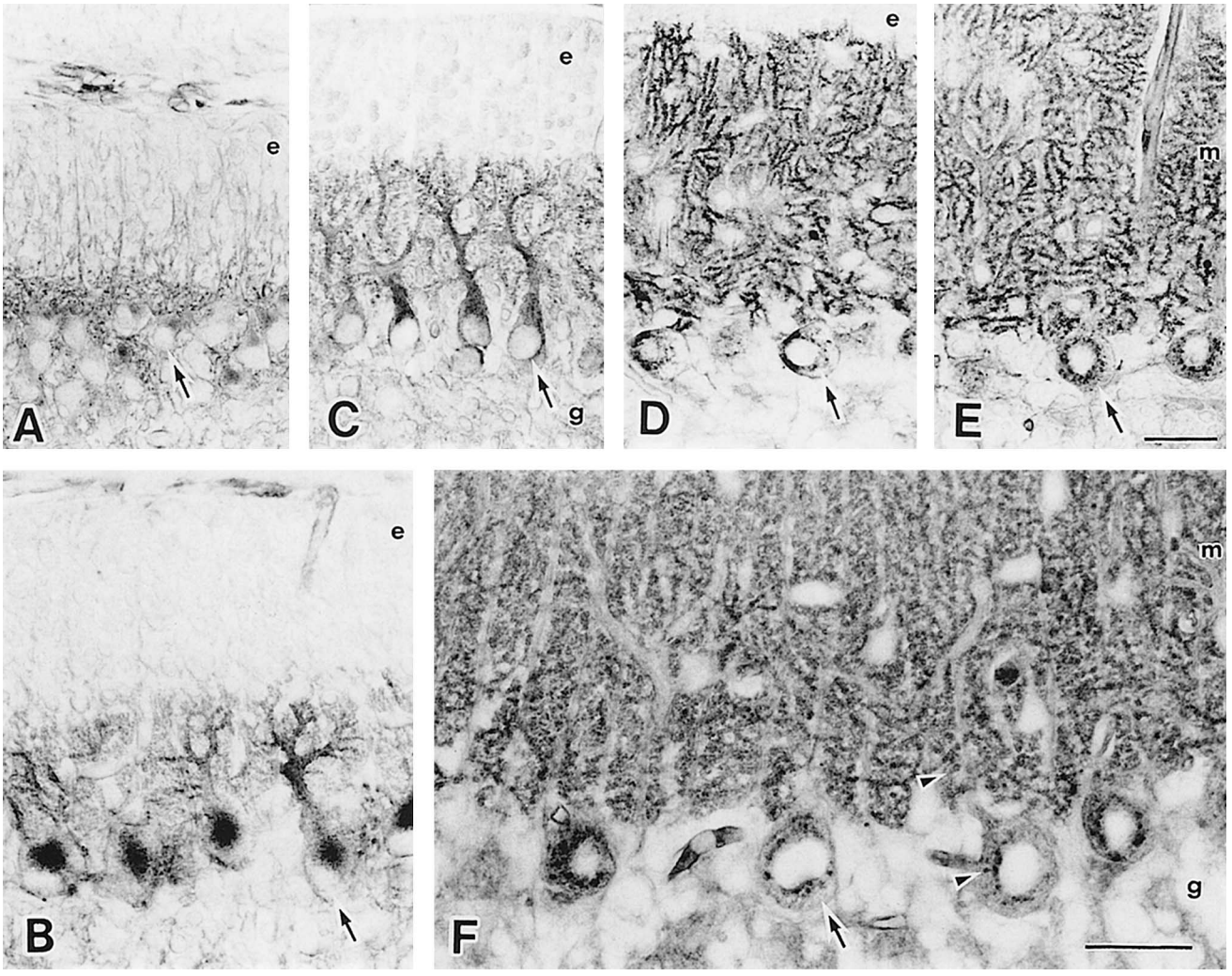

Fig. 3. Photomicrographs of rat cerebella stained with MAM. The granular structures in Purkinje cells (arrow) were strongly labeled with MAM at every developmental stage. The granular structures on dendrites strongly reacted with MAM on postnatal days 10 (**C**), 15 (**D**), and 20 (**E**), as did the dendrites of the adult rats (arrowhead). Abbreviations: e, external granule cell layer; m, molecular layer; g, granule cell layer. Bar, 30 μm.

The labeling distribution was not uniform on the dendrites of the cells. Spot-like labelings (Fig. 1B and C, small arrow) were observed on the surface of the dendrite.

Postnatal day 7 is equivalent to the initial stage at which Purkinje cells form synapses with parallel fibers (Altman, 1972). FGP7 may be synthesized at the onset of synaptogenesis. FGP7 may play at least one important role in the initiation of synapse formation. Zanetta *et al*. (1984,1985a, b) and Dontenwill *et al*. (1985) reported the transient appearance of glycoprotein(s) and lectin-like molecules in the rat cerebellum during the postnatal development. They proposed that the glycoprotein and the lectin-like molecules correspond to elements that recognize one another during the interactions between Purkinje cells and granule cells (Zanetta *et al*., 1985a, b). The glycoprotein is not identical to FGP7 because the location and timing of the appearance of the glycoprotein (Zanetta *et al*., 1975) are quite different from those of FGP7. Some lectins labeled parallel fibers of developed cerebellum, but there was no lectin which labeled the fibers at only the suckling period. Histochemical observations (Watanabe *et al*., 1996) revealed that anti-syndecan antibody combined with parallel fibers during the period when Purkinje cells showed strong combination ability to UEA-I. There are some possibilities that FGP7 fulfills the cell recognition functions with anti-syndecan antibody reactive glycoprotein (s).

We found little substantial information about FGP7. It would not combine with lipids because UEA-I and LTL strongly labeled Purkinje cells in paraffin sections, in which the cerebellum was thoroughly treated with organic solvents, such as chloroform, ethyl alcohol and xylene. In preliminary Western blot analysis, a few proteins had a strong ability to combine with UEA-I and LTL. They appeared in postnatal day 7 rat cerebellum but not in adult rat cerebellum. It seems probable that FGP7 combines with core protein. One such possible protein may be amphoterin which includes N-linked oligo sugar binding domains and transiently appears in the young

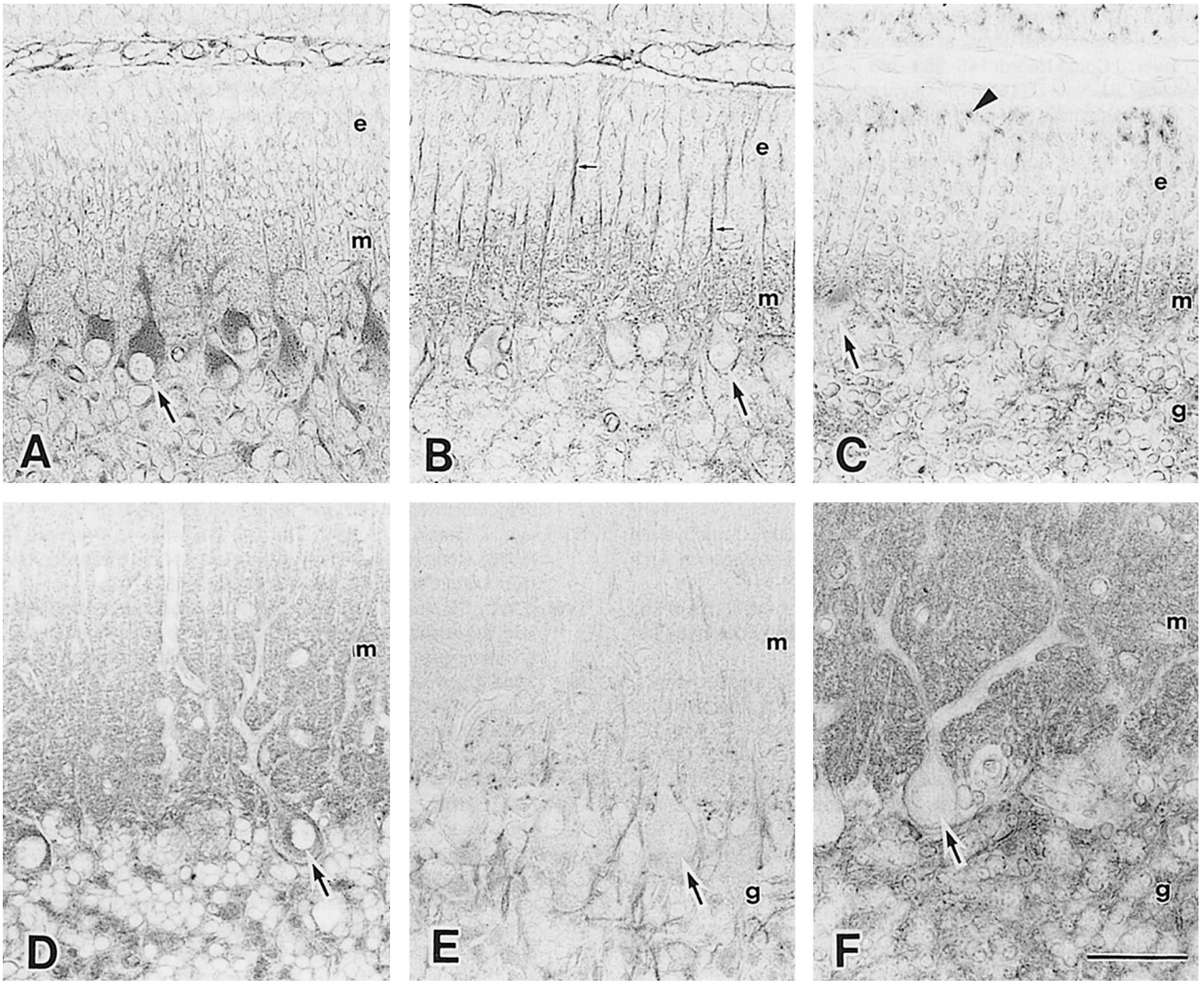

Fig. 4. Lectin histochemical demonstration of glycans in the cerebella. **A**, **B** and **C** show cerebella of postnatal day 7 rat. **D**, **E** and **F** show cerebella of adult rats. The cerebella of **A** and **D** were stained with Con-A. **B** and **E** were stained with DSL. C and F were stained with PNA. Purkinje cells (arrow) were strongly stained with Con-A. PNA and DSL faintly stained the Purkinje cells. Bergmann-glial fibers (small arrow) on postnatal day 7 were stained with DSL. Granular structures in external granule cell layers were strongly stained (arrowhead) with PNA. Abbreviations: e, external granule cell layer; m, molecular layer; g, granule cell layer. Bar, 30 μm.

rat brain (Merenmies *et al*., 1991). The core protein of FGP7 and the exact locations of sugar residues should be analyzed by more detailed biochemical and histochemical methods.

MAL reactive material(s) appeared in developed dendrites of Purkinje cells. It is known that some proteoglycans are expressed on the dendrites of cerebellar Purkinje cells of developed rats. Immunohistochemical observations have shown that phosphate-buffered saline-soluble brain proteoglycan is expressed on the dendrites of Purkinje cells in developed rats (Maeda *et al*., 1992). The 6B4 may be another glycan with MAL reactivity on the dendrites because the expression of 6B4 on the dendrites can not be detected by immunohistochemical methods on postnatal day 10 (Maeda *et al*., 1992). Synaptophysin is also expressed on the dendrites of Purkinje cells (Leclerc *et al*., 1989). Synaptophysin is a glycoprotein similar in immunohistochemical appearance to MAL reactivity on the dendrites of these cells. It is necessary to further examine MAL reactivity, with emphasis on the appearance of synaptophysin.

ACKNOWLEDGMENTS

Part of this study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, sports and Culture, Japan (No. 08670935). We would like to thank Mrs. M. Okada for technical assistance.

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(Received November 5, 1996 / Accepted November 6, 1997)