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Site restricted and neuron dominant expression of α2,8sialyltansferase gene in the adult mouse brain and retina

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Gene expression of the α2,8sialyltransferase (α2,8S-T) responsible for GD3 synthesis in the adult mouse brain and retina was analysed by reverse transcription-polymerase chain reaction/Southern blotting (RT-PCR/Southern) and *in situ* hybridization. Among various portions of the brain, high levels of 9.5 kb mRNA were observed in the retina and midbrain. Results of RT-PCR/Southern did not necessarily correlate with the enzyme activities in the individual sites. *In situ* hybridization analysis revealed that this gene was characteristically expressed in the inner segment of photoreceptor cells, some nuclei in the midbrain, cranial nerve nuclei in the pons-medulla, Purkinje cells in the cerebellum, pyramidal cells of the hippocampus and granular cells of the dentate gyrus. In the retina, the α2,8S-T gene was broadly expressed over the layers during development, and retained high expression levels in the photoreceptor cells of adult mice consistent with high expression of GD3. Destruction of neurons in the hippocampus and dentate gyrus by injection of kainic acid and colchicine respectively resulted in the disappearance of the hybridization signal, suggesting that the α2,8S-T gene was mainly expressed by neurons.

Keywords: α2,8sialyltransferase gene, mouse brain and retina, gene expression

Abbreviations: α2,8S-T, α2,8sialyltransferase; nu., nucleus (nuclei); mAb, monoclonal antibody; GFAP, glial fibrillary acidic protein; CNS, central nervous system; E, embryonic day; RT-PCR/Southern, reverse transcription-polymerase chain reaction/Southern blotting; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; nomenclature of gangliosides is based on that of Svennerholm [41].

Introduction

Among the gangliosides, sialic acid-containing glycosphingolipids, special attention has been paid to GD3 because the distribution of GD3 is very restricted and characteristic [1]. GD3 has been detected in undifferentiated or proliferating cells of neural cells, such as immature neuronal cells or reactive glial cells [2–5]. GD3 is also important as a tumour associated antigen [6]. Specifically, it is expressed on human malignant melanoma cells and cell lines [7], whereas normal melanocytes express GD3 at a minimal level [8]. These facts have enabled GD3 to be a good marker of malignancy or a target for immunotherapy [9]. GD3 has also been detected on cells transformed by the introduction of some oncogenes [10]. Furthermore, GD3 has been detected on activated T lymphocytes, while resting T cells express GD3 at a low level [11, 12].

Recently we and other groups isolated GD3 synthase (α2,8sialyltransferase) (EC 2.4.99.8) cDNA by an expression cloning system [13–15]. Using this cDNA as a probe we have analysed the expression of the GD3 synthase gene in various human cancer cell lines and T lymphocytes [16]. We have also analysed the expression

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of the gene in the mouse brain during development [17], and confirmed the contrasting expression pattern of the gene with that of the β1,4GalNAc transferase gene. Namely, expression of the GD3 synthase gene was very strong in the early stage of development but was then reduced gradually along with development as reported by Yu *et al.* [18]. However, some parts of the mouse brain showed persistent expression of the GD3 synthase gene even after birth. These facts suggested that GD3 is expressed at a fairly high level in restricted areas of the adult brain playing important roles in neuronal function.

In the present study, we have analysed the mRNA expression of the GD3 synthase gene in the adult mouse brain by using reverse transcription polymerase chain reaction/Southern blotting (RT-PCR/Southern) and *in situ* hybridization, and attempted to analyse the change in the gene expression pattern after treatment of the brain with neurotoxic reagents to identify the cells expressing the gene.

Materials and methods

Mouse

BALB/c mice were purchased from Japan SLC (Shizuoka, Japan) and housed in the Laboratory Animal Center for Biomedical Research, Nagasaki University School of Medicine. Embryos were obtained by mating male and female mice. Noon of the day on which the copulation plug was found was considered as embryonal 0.5 day (E0.5). Precise staging of dissected embryos was performed using *Kaufman's Atlas of Mouse Development* [19].

RNA isolation and Northern blotting

Cellular RNA was extracted as described previously [20]. Twenty μg of total RNA were separated on 1.2% agarose-formaldehyde gel, and the mRNAs were transferred onto a GeneScreen plus membrane (Dupont, Boston, MA). Hybridization with [α-^{32}P]dCTP-labelled mouse full-length α2,8S-T cDNA or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (nucleotides 255-1062) probes was performed as described [20], and radioactivity was visualized with an image analyser (Fuji Photo Film, BAS2000).

RT-PCR and Southern blotting

Single-strand cDNAs were synthesized using total RNA (3.5 μg) with the oligo $(dT)_{14}$ primer as described previously [21]. PCR was performed according to the previous report [21]. The PCR primers used for the α2,8S-T gene and for the GAPDH, were sense primer, 5′-AATGGTGGGATTCTGAA-3′, antisense primer, 5′-GATCTCTTTCTCGTTGGGC-3′and sense primer 5′-TGTCATCAACGGGAAGCCCA-3′, antisense primer, 5′-TTGTCATGGATGACCTTGGC-3′, respectively. Synthesized cDNAs were subjected to 29 and 23 cycles of amplification, for the α2,8S-T gene and GAPDH, respectively. RT-PCR products (5 μl) were separated on 10% polyacrylamide gel. Those of α2,8S-T and GAPDH were transferred onto a GeneScreen plus membrane. Hybridization with [α-^{32}P]dCTP-labelled mouse α2,8S-T cDNA (pD3T-m8) and GAPDH cDNA was performed as described [20], and then radioactivity was visualized with an image analyser.

α2,8S-T assay

The enzyme activity of α2,8S-T was determined according to the method described [22].

Riboprobe synthesis

Digoxigenine-labelled mouse α2,8S-T riboprobes were prepared by *in vitro* transcription of a BlueScript II SK- (Stratagene) containing mouse α2,8S-T cDNA (pD3T-m8) in the presence of digoxigenine-labelled UTP (Boehringer, Mannheim, Germany) as previously described [23].

In situ hybridization

In situ hybridization was performed as described [23]. After fixation and acetylation, hybridization was performed at 60 °C for 12–16 h. The sections were washed in 0.1 × SSC at 68 °C and treated with 20 μg ml^{-1} RNase A. Then slides were immersed in 0.5% blocking solution and incubated with 150 mU ml^{-1} polyclonal sheep anti-digoxigenin Fab-fragments conjugated to alkaline phosphatase. Colour development was performed according to the manufacturer's instruction (Boehringer, Mannheim). The names of the sites in the brain and retina were based on those in *The Rat Brain in Stereotaxic Coordinates* [24], in *The Rat Nervous System* [25] and *Kaufman's Atlas of Mouse Development* [19].

Colchicine and kainic acid injection

Mice were anaesthetized with 0.125 ml of 6 mg ml^{-1} pentobarbital (Pitman-Moore, NJ). Colchicine (Sigma, St Louis, MO) (3 μg in 0.5 μl saline) and kainic acid (Sigma) (0.25 μg in 0.5 μl saline) were injected into the dentate gyrus (1.5 mm lateral and 2.5 mm frontal to the lambda, and 2 mm below the pial surface) and the hippocampal CA3 pyramidal cell layer (2 mm lateral and 2.5 mm frontal to the lambda, and 2 mm below the pial surface) of 26–27 g weight BALB/c mice, respectively. The brains were isolated 7 days thereafter, and histologically analysed. All animals used for the procedures were treated in strict accordance with the NIH *Guide for Care and Use of Laboratory Animals* and approved by the Nagasaki University Animal Care Committee.

Antibodies

To detect astrocytes, we used anti-glial fibrillary acidic protein (GFAP) antibody (rabbit) purchased from Chemicon (Temecula, CA). To detect microglia cells we used anti-macrophage specific antigen (F4/80) antibody (rat IgG) and anti-Mac-1 (complement receptor, type III) antibody (rat IgG) [26]. Anti-F4/80 mAB was purchased from Serotec (Oxford, UK). Anti-Mac-1 mAb was produced by M18/2.a.8 hybridoma obtained from American Type Culture Collection.

Immunochemistry

Frozen tissue sections of 8 μm thickness were mounted on glass slides. After air-drying the sections were fixed for 10 min with 4% paraformaldehyde at room temperature. All following steps were performed at room temperature. Sections were washed with PBS (pH 7.0). Endogenous peroxidase was blocked by soaking the tissue sections in 0.6% H_2O_2 for 1 h. To avoid non-specific binding of the Ab, they were immersed in 5% BSA in PBS (blocking solution) for 30 min. Sections were incubated for 1 h with a primary Ab, diluted as follows: anti-GFAP, 1:100 dilution from serum; anti-F4/80, 1:20 dilution from culture supernatant; anti-Mac-1, 1:1000 dilution from ascites. After rinsing with PBS, sections were incubated with biotin-conjugated protein A (Zymed, San Francisco, CA) diluted at 1:50 (0.02 mg ml^{-1}) for 30 min for the detection of anti-GFAP Ab. The anti-F4/80 and anti-Mac-1 mAb were detected by biotin-conjugated anti-rat IgG (Vector, Burlingame, CA) diluted at 0.01 mg ml^{-1}. Following an additional rinse, sections were incubated in avidin-biotinylated horseradish peroxidase complex (Vector, Burlingame, CA) for 1 h. Horseradish peroxidase activity was detected using 0.4 mg ml^{-1} diaminobenzidine with 0.01% H_2O_2 in 0.1 M Tris-HCl (pH 7.2) for 5 min. Colchicine treated samples were counter stained with Hematoxylin and kainic acid treated samples were not.

Results

EXPRESSION OF α2,8S-T GENE IN THE CNS (CENTRAL NERVOUS SYSTEM)

By Northern blotting, expression of the α2,8S-T gene was analysed in the whole cerebrum and cerebellum (Fig. 1A). The result revealed that the mRNA was strongly expressed in these tissues and the size of the mRNA was 9.5 kb. To examine the expression of α2,8S-T mRNA in the CNS, we performed RT-PCR for nine different parts of the adult mouse CNS and retina (Fig. 1B), although this method is not fully quantitative. This mRNA was abundantly expressed in retina and midbrain. Moderate level of the gene expression was detected in the cortex, hippocampus, striatum, thalamus, pons-medulla and cerebellum. Weak but definite bands were detected in the spinal cord and olfactory bulb. Relative intensities of the bands were compared after correction with those of GAPDH mRNA, and summarized in Fig. 2.

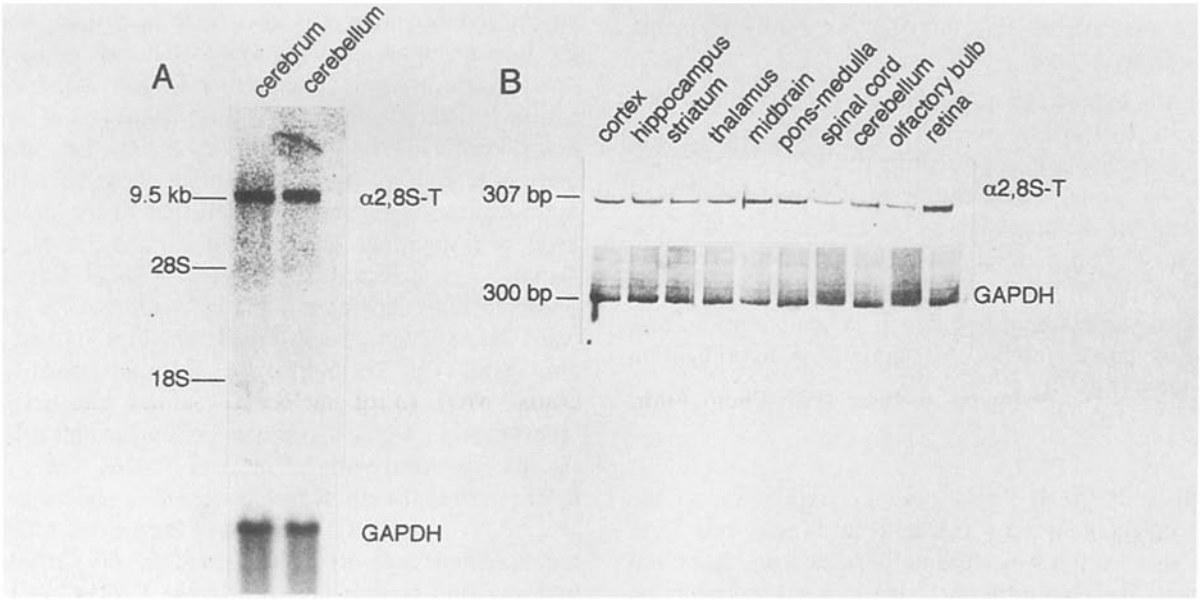

Figure 1. The expression of the α2,8S-T gene in various parts of adult mouse CNS tissues and retina. (A) Five μg of poly (A)$^+$ RNAs were applied to an agarose-formaldehyde gel, blotted, and hybridized with mouse α2,8S-T cDNA or mouse GAPDH cDNA as described in Materials and methods. (B) RT-PCR was performed with 3.5 μg total RNAs and quantitated by Southern blot of its products as described in Materials and methods. Tissues from which the RNA were isolated are shown at top of the figure. The sizes of bands are shown at the side.

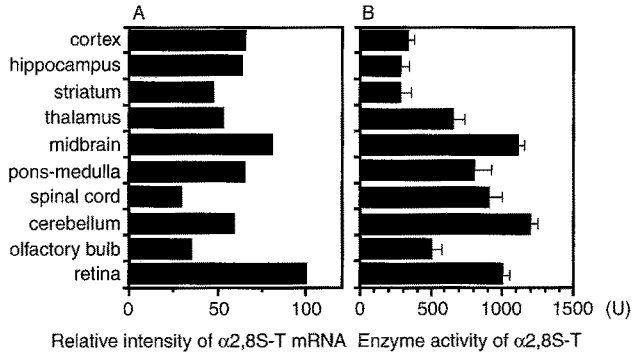

Figure 2. The expression of the α2,8S-T gene and enzyme activity in the adult mouse CNS. Relative amount of the mRNA was determined by imaging analyser on Southern blot membrane in Fig. 1. Relative amounts of the mRNAs were presented as percent of that of the retina. α2,8S-T enzyme activity was determined by an enzyme assay with three aliquots as described in Materials and methods. The mean volumes ± SE are shown.

ENZYME ACTIVITY OF α2,8S-T GENE IN THE CNS

Enzyme activities of α2,8S-T in individual parts of CNS were also examined. High activity areas (more than 1000 U, pmol per mg protein h^{-1}) were midbrain and cerebellum. Moderate activity areas (1000–500 U) were thalamus, pons-medulla, spinal cord, olfactory bulb and retina. Low activity areas (less than 500 U) were cortex, hippocampus and striatum. Enzyme levels of α2,8S-T in individual parts are summarized together with relative intensities of α2,8S-T mRNA in Fig. 2.

THE DISTRIBUTION OF CELLS POSITIVE FOR THE α2,8S-T mRNA IN THE CNS

Using *in situ* hybridization, we investigated the distribution of cells expressing the mRNA of this gene in the CNS. In particular we analysed in detail the expression pattern in the retina, where this gene is expressed at high levels during the development as well as in the adult. The tissues that showed a unique expression pattern are also described in detail below. Expression of the mRNA in other areas is summarized in Table 1. When we used sense probe for negative control, no signals of hybridization were detected (Fig. 3I).

Retina

In the retina of the E12 embryo, the expression of the gene was detected broadly in the neuroblastic cell layer (Fig. 3B). In E18, the first differentiated neurons, ganglion cells, from the neuroblastic cell layer as well as neuroblastic cell layer were labelled similarly (Fig. 3D). In the adult retina, the inner segment of photoreceptor cells were strongly labelled and the outer nuclear layer were weakly labelled (Fig. 3F and 3H). The inner nuclear layer and ganglion cell layer were labelled moderately but these signals were very clear. In the inner and outer plexiform layers, no hybridization signal was detected.

Midbrain

Strong hybridization signals were detected in the red nucleus, substantia nigra (Fig. 4B) and occulomotor nucleus (nu.) (data not shown). Weak but significant signals were detected in the coliculus complex, dorsal raphe nu. and medial raphe nu. (data not shown).

Pons-medulla

Strong signals were detected in the pontic nu. and cranial nerve nu., including the motor trigeminal nu., facial nu., vestibural nu. and lateral reticular nu. (Fig. 4D, 4F and 4H).

Cerebellum

Clear signals were detected on the Purkinje cells (Fig. 4J). Weak signals were on the granular cells and deep nu. No signal was detected on the molecular layer or white matter.

Hippocampus region

Moderate hybridization signals were detected on CA1-CA4 pyramidal cells of hippocampus and granular cells of dentate gyrus (Fig. 5B). No signal was detected on the lacunosum molecular layer, oriens layer, fibria or radiatum layer.

EFFECTS OF COLCHICINE AND KAINIC ACID ON THE EXPRESSION OF THE α2,8S-T mRNA

In situ hybridization was also performed using sections of the hippocampus lesioned with colchicine or kainic acid. After intracerebral injection of colchicine, granular neurons of the dentate gyrus were destroyed as previously described [27] (Fig. 5F and 5J). On the other hand, pyramidal cells of the hippocampus were not affected. A hybridization signal was not detected in the granular cell layer with neuronal damage (Fig. 5F and 5J). No apparent signals were detected in the sites of local gliosis, which occurred after destruction of the neurons. The infiltrated cells were stained by anti-GFAP Ab (Fig. 5G and 5K) and anti-F4/80 (Fig. 5H and 5L) or anti-Mac-1 mAb (data not shown), indicating that they were of glia cell lineage. Hybridization signals in other areas were not affected. In the mice injected with kainic acid [28 29], CA1, CA3 and CA4 pyramidal cells were destroyed as shown in Fig. 6A and 6B, and many GFAP-positive astrocytes infiltrated in the lesioned area as shown in Fig. 6C. In this area, hybridization signals detected in the CA1, CA3 and CA4 pyramidal cells disappeared (Fig. 6E), although they remained in the CA2 pyramidal cells and granular cells of dentate gyrus which were relatively resistant to kainic acid. No definite signals were found in the reactive glia cells expressing GFAP or F4/80 (Fig. 6C and 6F).

Table 1.

Sites	*Expression of* α2,8S-T mRNA*
Olfactory bulb	
Glomerular cell	+
Mitral cell	++
Ant. olfactory nu.	+
Allocortex	
Piliform cortex and entorhinal cortex	+
Isocortex	
Layer V	+
Hippocampus	
Pyramidal cell (CA1–CA4)	++
Granular layer (DG)	++
Striatum	
Lateral and dorsomedial septal nu.	+
Braca	+
Hypothalamus	
(Paraventricular nu., supraoptic nu., suprachiasmatic nu., dorsomedial nu., and ventromedial nu.)	+
Medial and lateral mammillary nu.	++
Thalamus	
Anterodorsal nu. reticular nu. ventrolateral nu. Subthalamic nu. ventroposterior nu., Lateral hanbenular nu., zona incertant	++
Dorsolateral and medial geniculate nu.	+
Amygdala	
Basolateral amygraroid nu.	+
Midbrain	
Colliculus superior, inferior	+
Substantia nigra	++
Red nu.	+++
Dorsal and medial raphe nu.	+
Principal occulomotor nu.	++
Pons	
Pontic nu.	++
Medulla	
Corporis trapezoidei	+++
Facial nu.	+++
Mortor trigeminal nu.	+++
Lateral reticular nu.	+++
Lateral supra olive	+
Cerebellum	
Purkinje cell	++
Granular cell	+
Deep nu.	+
Retina	
Ganglion cell	+
Outer nuclear cell	+
Inner nuclear cell	++
Inner segment of photoreceptor cell	+++

*Expression of α2,8S-T mRNA was determined by *in situ* hybridization as described in Materials and methods. Based on relative optical density measurements, the signal was scored as weak (+), moderate (++), or strong (+++).

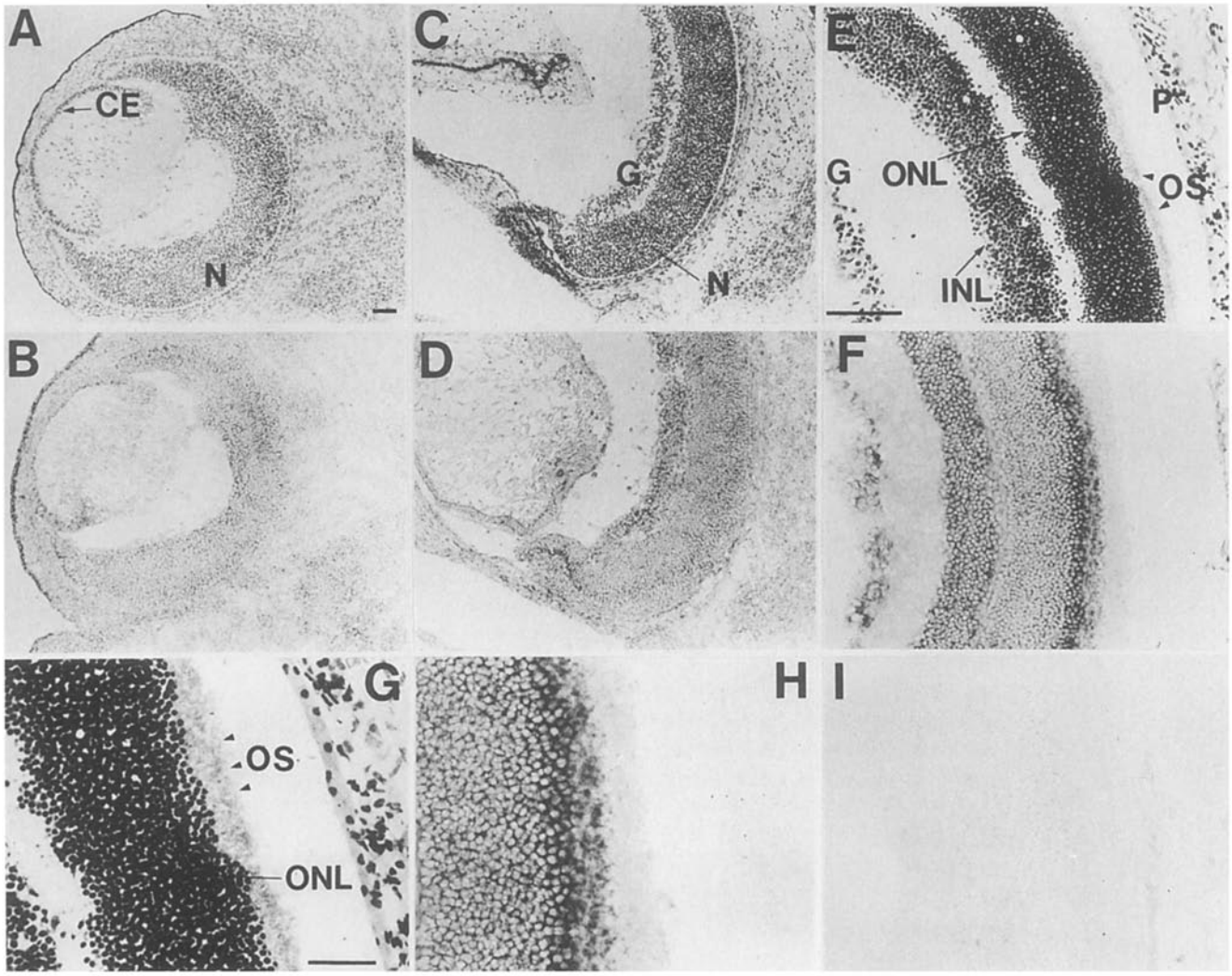

Figure 3. The expression of the α2,8S-T gene in the retina. *In situ* hybridization was performed using digoxigenin-labelled α2,8S-T cRNA probes as described in Materials and methods. A, C, E and G were Hematoxilin-eosin stain (HE). B, D, F and H were hybridized with an antisense probe. I was hybridized with sense probe. A and B were sections of the E12.5 C and D were E18.5. E-I were postnatal 10 weeks. G, ganglion cell layer; INL, inner nuclear cell layer; ONL, outer nuclear cell layer; IS, inner segments of photoreceptor cell; P, pigment cell layer. The magnifications of A–D were the same. That of G and H, and E, F and I were also the same. Bar in A and E represents 100 μm. Bar in G represents 50 μm.

Discussion

Since an anti-GD3 mAb became available, expression and distribution of GD3 in the CNS have been analysed by immunohistochemical techniques [5, 30, 31]. However, it has not been demonstrated what kind of cells are synthesizing the GD3 synthase enzyme and GD3 ganglioside. In the present study, we demonstrated that the expression of the gene was relatively predominant in the retina and midbrain in the adult mouse CNS. When the expression of the mRNA was analysed by *in situ* hybridization, strong signals were detected in the inner segment of photoreceptor cells of the retina and red nu. in the midbrain and some nu. of the cranial nerves in the medulla. Although biochemical analysis has revealed the predominance of α2,8S-T in early neuronal development [18], and a decrease in the activity in the late stage of development, our results demonstrated diverse expression levels of the gene depending on the parts of the CNS [17]. Actually, the activity of the gene was conserved at a high level in some parts of the adult CNS, i.e. retina and midbrain showed high enzyme activity (more than 1000 U). The enzyme levels in some parts of the brain, however, did not necessarily correlate with the mRNA levels. Especially, the enzyme levels of the spinal cord and cerebellum were much higher than expected from their

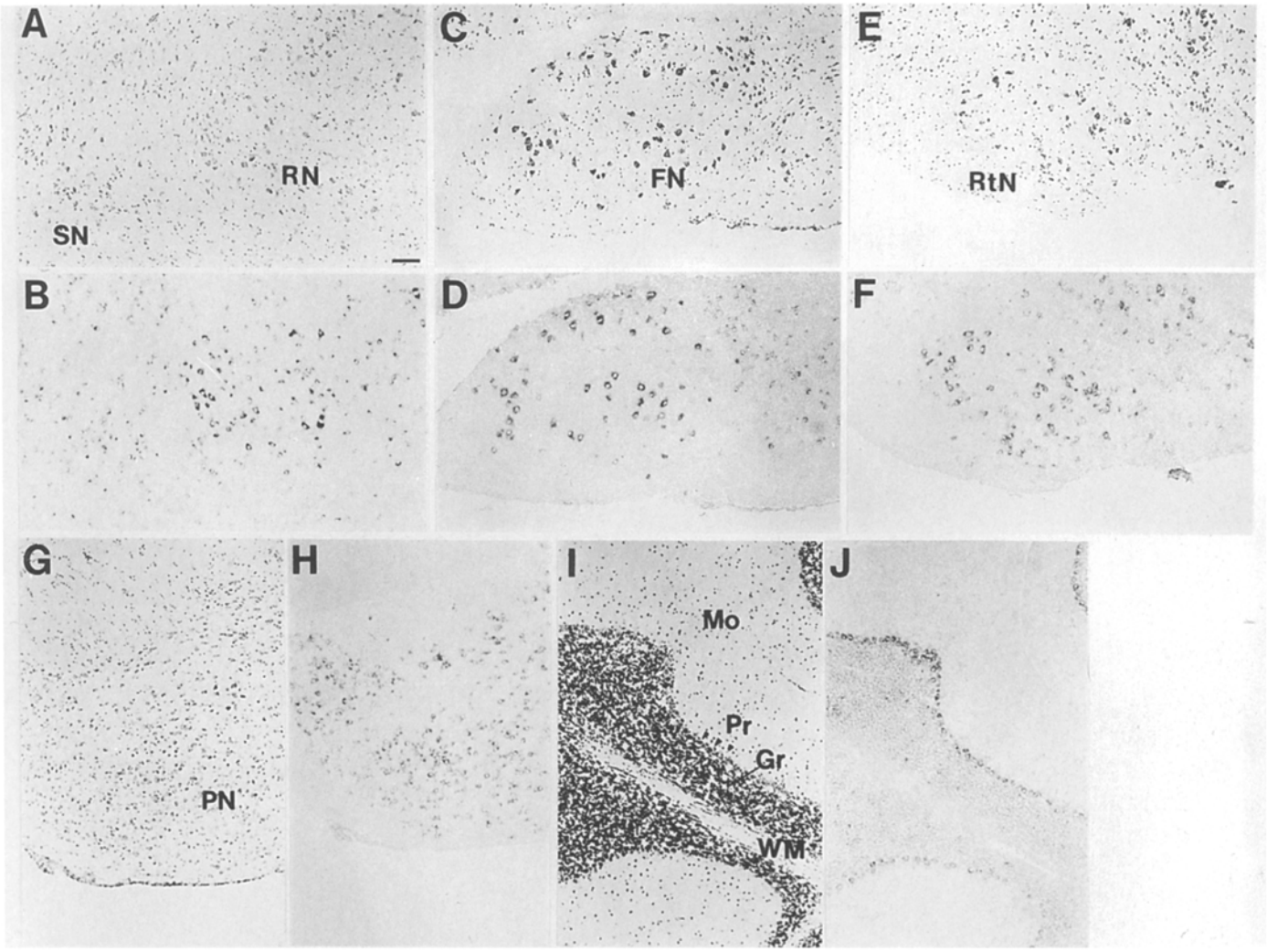

Figure 4. The expression of α2,8S-T gene in the midbrain, pons, medulla and cerebellum. *In situ* hybridization was performed as described in Fig. 3. A, B, G and H are frontal sections. C, D, E, F, I and J are sagital sections. A, C, E, G and I were Nissle stain. B, D, F, H and J were hybridized with the antisense probe. RN, red nucleus; SN, substantia nigra; FN, facial nucleus; RtN, reticular nucleus; PN, pontic nucleus; Mo, molecular layer; Gr, granular layer; WM, white matter; Pr, Purkinje cell layer. Bar in A represents 100 μm.

mRNA levels. The discrepancy between mRNA levels and the enzyme activities in certain areas may be explained by various factors. It may be partly because the enzyme activity is being regulated at the post-translational level i.e. by phosphorylation/dephosphorylation of some sialyltransferases [32] or glycosylation as described previously [33]. Another reason might be the intracellular transport of the enzyme molecules along dendrites and/or axons of neurons synthesizing the mRNA. It may also be possible that there exists another GD3 synthase, which cannot be detected by the probe used here.

There have been may reports to suggest that GD3 is characteristically expressed in undifferentiated or proliferating cells [1]. GD3 has also been claimed to be expressed in reactive glia cells [2, 3] or transformed cells [10] reflecting the cellular activity. In our recent study, it was also demonstrated that the GD3 synthase gene was expressed mainly in the early developmental stage of mouse CNS, and was gradually reduced with development when analysed in brain tissue as a whole mass [17]. Although some parts of the adult brain showed sustained expression of the GD3 synthase gene, many of them co-expressed the GM2/GD2 synthase gene, indicating that the final products are more complex gangliosides than GD3. On the other hand, high expression of GD3 [34, 35] or of GD3 synthase activity [36, 37] in retina has been observed. This corresponded with high expression of the GD3 synthase gene in our study. Strong signals were detected in the inner segment of the photoreceptor cells in retina. Daniotti *et al.* reported that adult rat retina

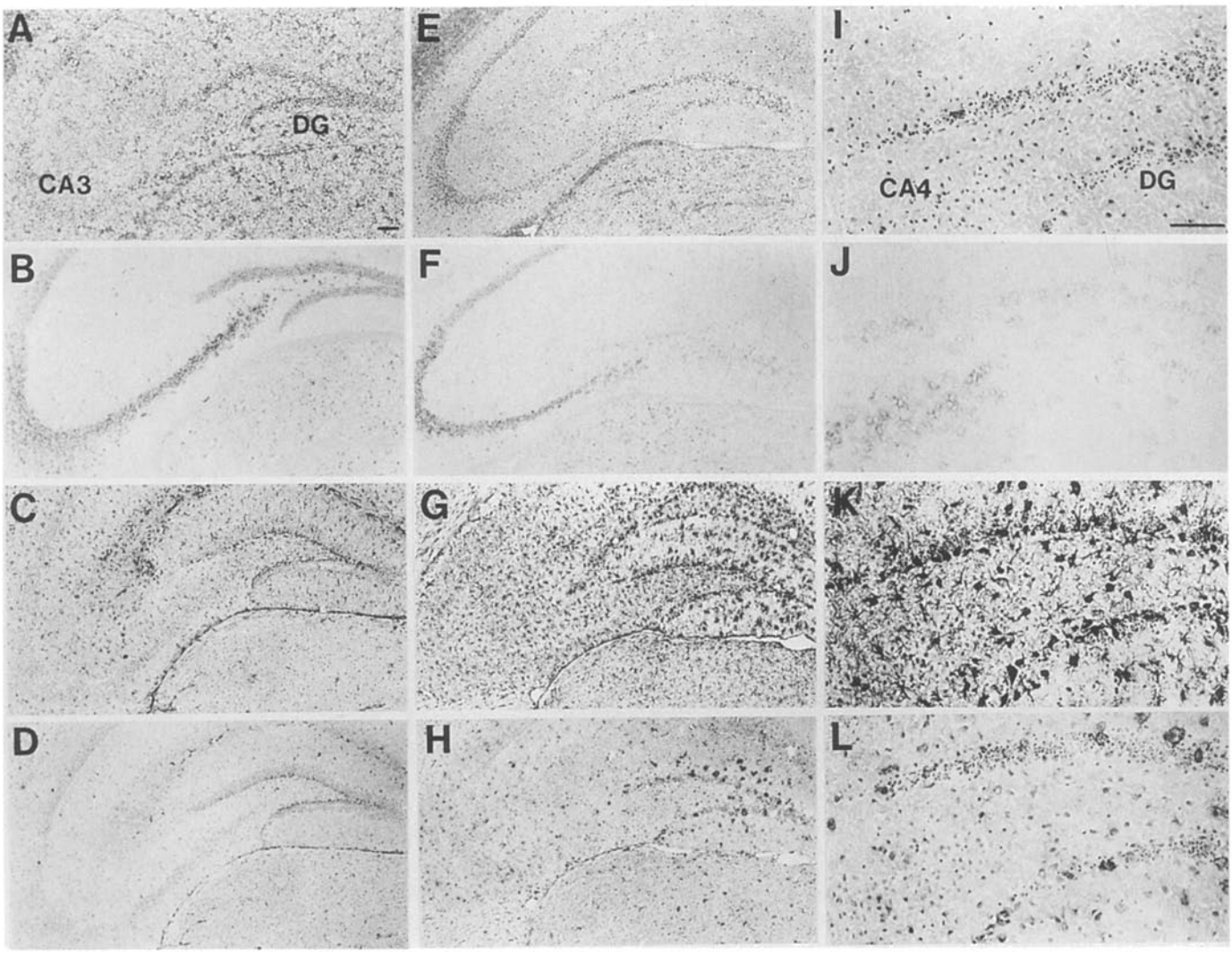

Figure 5. The effects of colchicine treatment on the expression of the α2,8S-T gene. Colchicine was directly injected into the dentate gyrus as described in Materials and methods. Seven days later, brains were isolated and analysed for expression of the α2,8S-T gene and glia cell markers by means of *in situ* hybridization and immunohistostaining, respectively, as described in Materials and methods. All samples are frontal sections. E–L were specimens injected with colchicine. A–D were non-injected side of the same sample. A, E and D were stained with HE. B, F and J were hybridized with the antisense probe. C, G and K were stained with anti-GFAP Ab. D, H and L were stained with anti-F4/80 mAb. A–D were printed inversely. CA3 and CA4, areas of hippocampus pyramidal layer; DG, dentate gyrus. Bar in A and I represents 100 μm.

interneurons, not glia cells, synthesize GD3 [37]. There have also been reports that *O*-acetylated GD3 was characteristically expressed during the development of rat retina [38, 39]. Varki *et al.* [40] demonstrated the importance of carbohydrate with 9-*O*-acetyl-sialic acids (possibly a GD3 derivative) in the organogenesis of the retina lamina structure using transgenic mice. Taken together with these previous reports and the location of the strong signal of the hybridization in the retina, it was strongly suggested that expression of α2,8S-T in the retina was essential for the development and function of the retina through life.

In situ hybridization analysis of the gene revealed that cells positive for this gene were neuronal cells. Previous reports indicated that GD3 ganglioside was a glia cell marker [3–5] and the expression of GD3 was induced at sites of local gliosis. The *in situ* hybridization analysis for the lesioned tissues demonstrated controversial results. In local gliosis, hybridization signals disappeared with neuronal damage induced by cochicine or kainic acid treatment. In the glia cells expressing GFAP and/or F4/80, a hybridization signal was not detected. The discrepancy between our results and previous reports on ganglioside synthesis in glia may be explained as follows. First, α2,8S-T expression in the glia may be lower than the level detectable with the *in situ* hybridization assay;

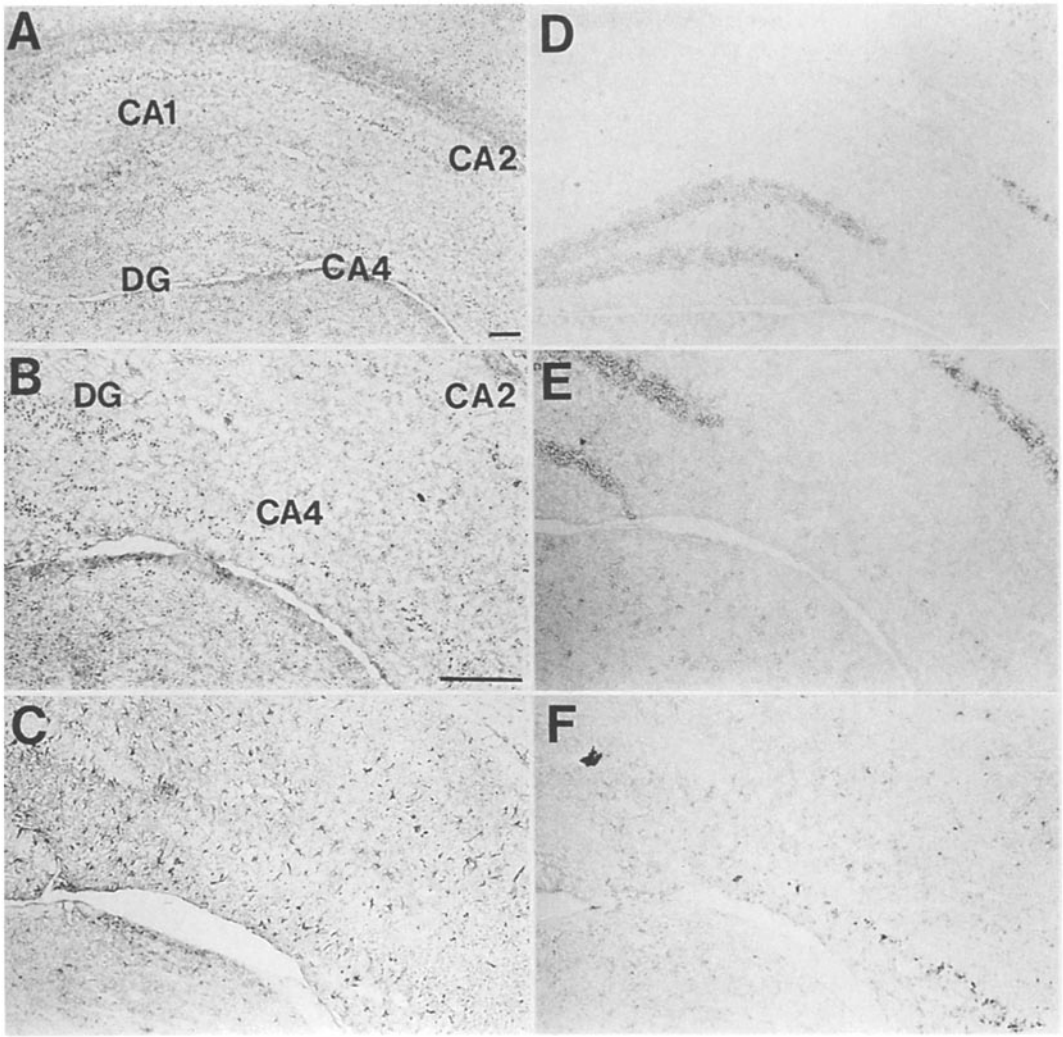

Figure 6. The effects of kainic acid treatment on the expression of the α2,8S-T gene. Kainic acid was directly injected into the hippocampal CA3 region as described in Materials and methods. Seven days later, brains were isolated and expression of the α2,8S-T gene and glia cell markers were analysed by means of *in situ* hybridization and immunohistostaining, respectively as described in Materials and methods. All samples are frontal sections. A and B were stained with HE. D and E were hybridized with the antisense probe. C was immunohistostaining with anti-GFAP Ab. F was immunohistostaining with anti-F4/80 mAb. CA1–4, areas of hippocampus pyramidal layer; DG, dentate gyrus. Bar in A and B represents 100 μm.

second, there may be another pathway for ganglioside synthesis; third, intercellular transport of gangliosides may occur. We are now trying to clarify this point.

Acknowledgements

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