

## Review

### A group of glycosphingolipids found in an invertebrate: Their structures and biological significance

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**Abstract:** A novel group of glycosphingolipids was identified in the nervous tissue and skin of the mollusc, *Aplysia kurodai*, which lacks gangliosides. More than 30 glycolipids were detected on HPTLC plates and the structures of 9 major glycolipids were determined. They were pentaosylglycosphingolipids and their common core structure was GalNAc $\alpha$ 1 $\rightarrow$ 3Gal $\beta$ 1 $\rightarrow$ 4Glc $\beta$ 1 $\rightarrow$ 1ceramide, except for one glycolipid in which Gal $\beta$  of the core structure was replaced by Gal $\alpha$ . 3-*O*-MeGal $\beta$  or 4-*O*-MeGlcNAc $\alpha$  or 3,4-*O*-carboxyethylideneGal $\beta$  was at their non-reducing ends. Gal $\alpha$  or Fuca binds to Gal of the core structure at 2C as a side chain sugar. One to three 2-aminoethylphosphonic acids and/or phosphoethanolamine link to the glycolipids. Immunohistochemically, glycolipids having carboxyethylideneGal at their non-reducing ends were localized exclusively in nerve bundles. Glycolipids activated cAMP-dependent protein kinase in the rat brain and may directly activate cAMP-dependent protein kinase in a manner similar, but not identical, to that of cAMP. The biological functions of glycolipids may share neurobiological functions proposed for gangliosides in vertebrates.

**Keywords:** glycosphingolipids, *Aplysia kurodai*, chemical structures, tissue distribution, neurobiological functions

#### 1. Introduction

In 1966, we reported our first method for the isolation of many neuronal cell bodies from the rat brain.<sup>1)</sup> This method, and that by Rose,<sup>2)</sup> prevailed widely. However, electron-microscopically, cell bodies were not well preserved for the analysis of neuronal cell membranes. Therefore, we invented a second method in which Ficoll solution was used in place of an acetone-glycerol mixture as the isolation medium

and a pig brain stem was used in place of a rat brain as the source of neuronal cell bodies,<sup>3)</sup> and by using standard routine biochemical methods, we could identify many biochemical characteristics of the neuron.<sup>4)</sup> The content of gangliosides was similar to or higher than that of other reports in which neuronal cell bodies were isolated in bulk, but lower than that of the grey matter of the pig cerebral cortex<sup>5)</sup> and hand-dissected neuronal cell bodies isolated from Deiter's vestibular nucleus of the ox.<sup>6)</sup> Electron-microscopically, the preservation of neuronal cell membranes isolated by our second method varied from experiment to experiment, and we could not improve on the method to constantly preserve neuronal cell membranes.

Gangliosides have long been considered to be enriched in membranes of the neuron and are indispensable for neuronal functions.<sup>7),8)</sup> In this context, we began to analyze, neurophysiologically well documented nerve tissues as a whole without isolating neuronal elements from these tissues. We analyzed the ganglia and nerve fibers of the sea hare, *Aplysia kurodai*, and giant nerve fibers of the

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Abbreviations: HPTLC: high-performance thin-layer chromatography; GC-MS: gas chromatography-mass spectrometry; FAB-MS: fast atom bombardment-mass spectrometry; PMR: proton magnetic resonance spectroscopy; 2-AEP: 2-aminoethylphosphonic acid; cer: ceramide; HF: hydrogen fluoride; -OME: methoxy; MAP2: microtubule-associated protein 2; MBP: myelin basic protein; PKC: protein kinase C; cAMP-kinase: cyclic AMP-dependent protein kinase.

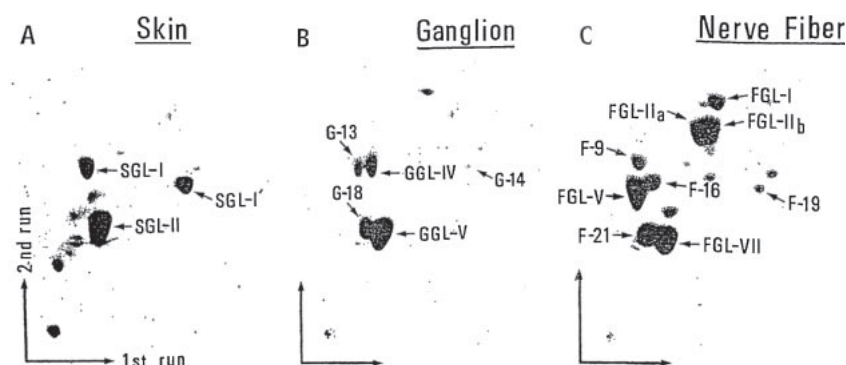


Fig. 1. Two dimensional HPTLC of water-soluble lipid fractions from the skin (A), ganglia (B), and nerve fibers (C) of *Aplysia kurodai*. Lipids were located with anthrone reagent.

crayfish, *Cambarus clarki*. Gangliosides were not detected, but several water-soluble phosphorus-containing lipids were identified in these nerve tissues.<sup>9),10)</sup>

## 2. Structures of water-soluble phosphorus-containing lipids from the tissues of *Aplysia kurodai*

Water-soluble phosphorus-containing lipids were extracted with a chloroform-methanol-water mixture from fresh or frozen ganglia, nerve fibers, and acetone-powder of the skin of *Aplysia kurodai* caught at Sado Island, separated by phase separation, and then purified by Sephadex column chromatography. Two-dimensional chromatograms of water-soluble lipid fractions from these three tissues are shown in Fig. 1. Most of the glycolipids detected by anthrone reagent were also stained with fluorescamine reagent for amines, and stained rather weakly with Dittmer-Lester's reagent for phosphorus.<sup>11)</sup> The two-dimensional thin-layer chromatographic patterns of the three tissues were very reproducible and we assumed that three main spots of the skin, SGL-II, SGL-I, and SGL-I' (Fig. 1A), may be the same glycolipids as the three glycolipids of ganglia, GGL-V, GGL-IV, and G-14 (Fig. 1B), and three glycolipids of nerve fibers, FGL-VII, F-16, and F-19 (Fig. 1C), respectively. The G-18 and G-13 glycolipids of ganglia may be the same as F-21 and FGL-V of nerve fibers, respectively. Glycolipids FGL-IIa, FGL-IIb, F-9, and FGL-I appeared to be specifically concentrated in nerve fibers. Immunochemical studies<sup>12)-14)</sup> support our assumption. In this context, we determined the structures of nine glycolipids, SGL-II, SGL-I, and SGL-I' of the skin and F-9, F-21, FGL-I, FGL-IIa, FGL-IIb, and FGL-V of the nerve fibers of *Aplysia*.

Glycolipids were isolated and purified as described previously.<sup>14)-22)</sup> In short, acetone powder of the skin or fresh nerve fibers of *Aplysia* was extracted with chloroform-methanol-water (30:60:10, v/v) and purified by two consequential silica gel columns monitoring their purities on HPTLC plates. Their structures were determined by sugar analysis, amino acid analysis, permethylation studies, fast atom bombardment-mass spectrometry, and proton magnetic resonance spectroscopy.

The chemical structures of the nine sphingoglycolipids are shown in Fig. 2. As described above, three of them, SGL-II, SGL-I, and SGL-I', isolated from the skin may also exist in ganglia and nerve fibers (Fig. 2a). Two glycolipids, F-21 and FGL-V, isolated from nerve fibers also may exist in ganglia (Fig. 2b). Four pyruvylated glycolipids, F-9, FGL-IIa, FGL-IIb, and FGL-I, seem to be unique to nerve fibers (Fig. 2c).

The structural characteristics of the nine sphingoglycolipids are summarized as follows. ① They are pentaosylglycosphingolipids. ② The common core structure of these glycosphingolipids is  $\text{GalNAc}\alpha 1 \rightarrow 3\text{Gal}\beta 1 \rightarrow 4\text{Glc}\beta 1 \rightarrow 1\text{ceramide}$ , except for F-21 in which  $\text{Gal}\beta$  of the core structure is replaced by  $\text{Gal}\alpha$ . ③ As a non-reducing terminal sugar, 3-*O*-MeGal $\beta$ , 4-*O*-MeGlcNAc $\alpha$ , or 3,4-*O*-(*S*-1-carboxyethylidene)Gal attaches to the core structure. Five glycosphingolipids having pyruvylated Gal as their terminal sugar may be fairly acidic. ④ As a side chain sugar,  $\alpha$ -Fuc or  $\alpha$ -Gal links to C2 of Gal of the core structure. ⑤ Glycosphingolipids contain phosphorus and amine as 2-aminoethylphosphonic acid or phosphoethanolamine and are zwitterionic. One to three moles of 2-aminoethylphosphonic acid (2-AEP) and/or one mole of phosphoethanolamine link to the glycosphingolipids. 2-AEP is characterized by a C-P

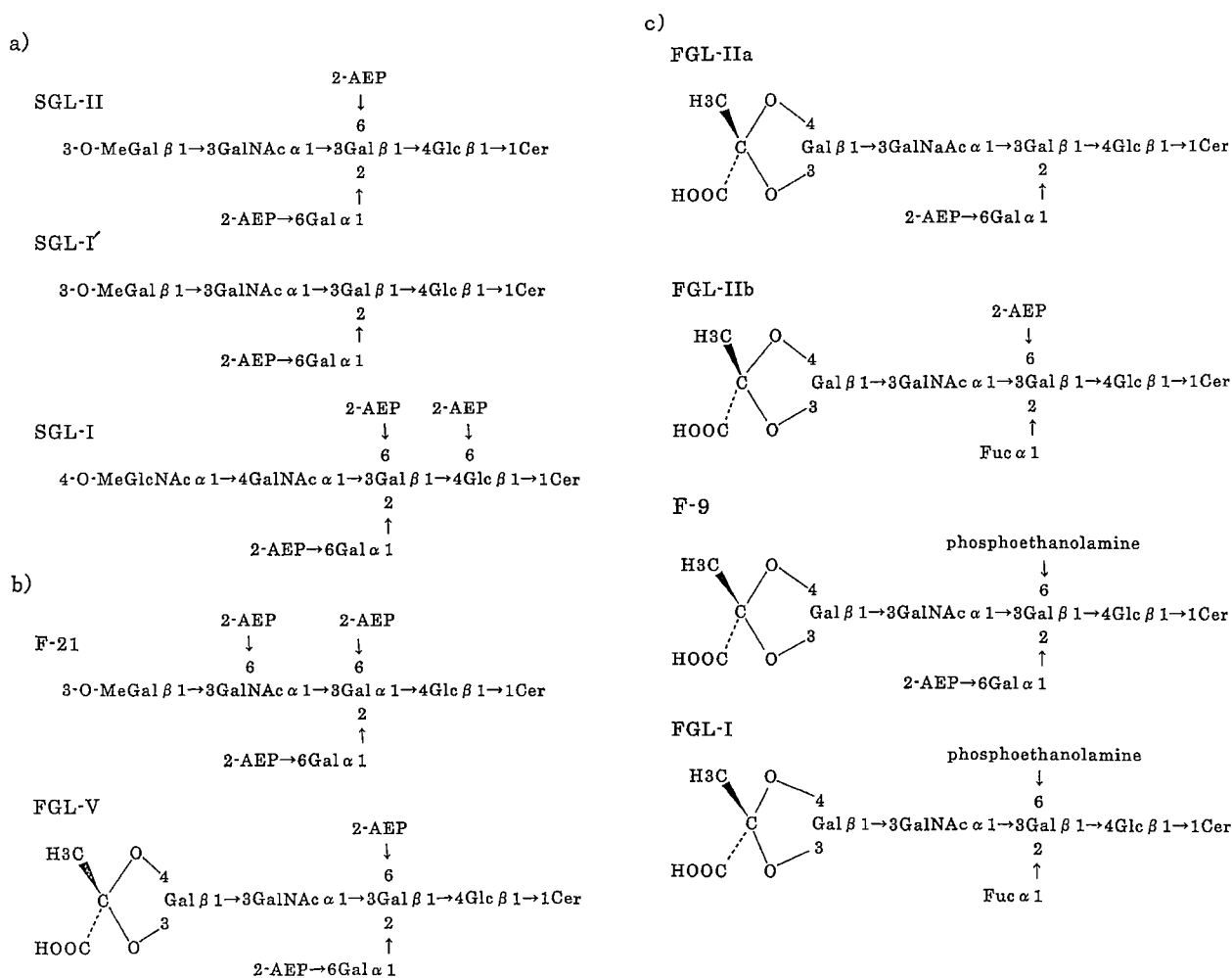


Fig. 2. Structures of 9 glycosphingolipids isolated from the mollusc, *Aplysia kurodai*. a) These three glycolipids seem to be present in the skin, ganglia, and nerve fibers. b) These two glycolipids seem to be present in ganglia and nerve fibers. c) These four glycolipids seem to be present exclusively in nerve fibers.

bond. ⑥ 2-AEP links to the side chain of Gal and/or directly to Gal, Glc, or GalNAc of the core structure at C6 of those sugars. Phosphoethanolamine links to C6 of Gal of the core structure. ⑦ As for the aliphatic portion of glycosphingolipids, the major fatty acid is palmitic acid, except for SGL-II and F-21, which have stearic acid as the second major fatty acid. Two major sphingosine bases of the glycolipids are octadeca-4-sphingenine and anteisnonadecasphingenine.

It is interesting that F-21, which exists in the nervous system at high concentrations, is supposed to have a specific conformation formed by intramolecular interactions in sugar moieties. Two or three of the 2-AEP groups may serve as a chelating site for cations or anions.<sup>20)</sup>

### 3. Immunochemical characteristics of glycosphingolipids and their distribution in the nervous tissues of *Aplysia kurodai*

Antisera were raised in rabbits using three glycolipids, SGL-II, SGL-I, and FGL-IIb, those having differently modified sugars, 3-*O*-MeGal, 4-*O*-MeGlcNAc or 3,4-*O*-(*S*-1-carboxyethylidene)Gal, at their non-reducing ends. The specificities of these antisera were certified on thin-layer chromatograms.<sup>12)-14)</sup> For immunohistochemical studies, freshly dissected abdominal ganglia and nerve fibers of *Aplysia kurodai* were stained by the indirect immunoperoxidase method.<sup>12),13)</sup>

The antiserum against SGL-II stained glycolipids with 3-*O*-MeGal at their non-reducing ends on

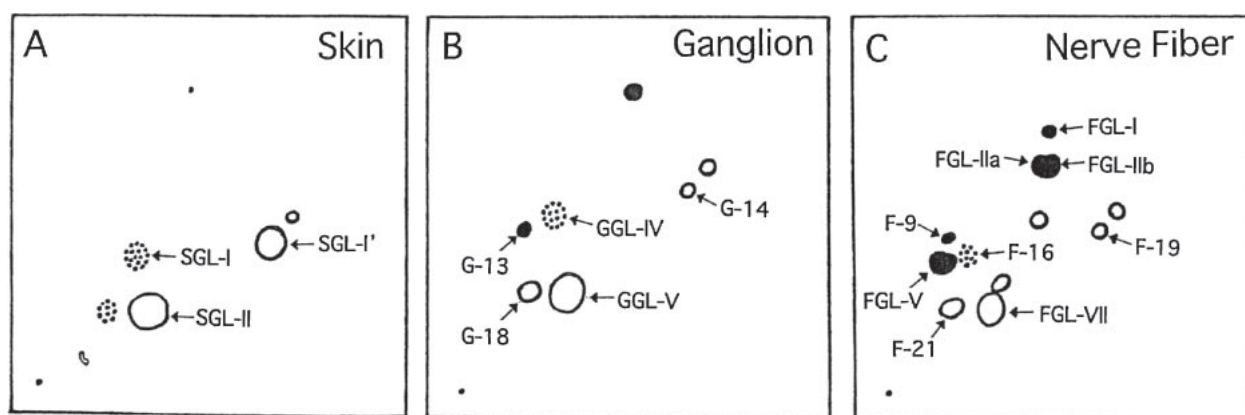


Fig. 3. Diagrammatic representation of radioimmuno-thin-layer chromatograms of total glycolipids isolated from the skin (A), ganglia (B), and nerve fibers (C) of *Aplysia kurodai*. Plain circles indicate glycolipids that reacted to the antiSGL-II antiserum, dotted circles indicate glycolipids reactive to the antiSGL-I antiserum, and dark circles indicate glycolipids that reacted to the antiFGL-IIb antiserum.

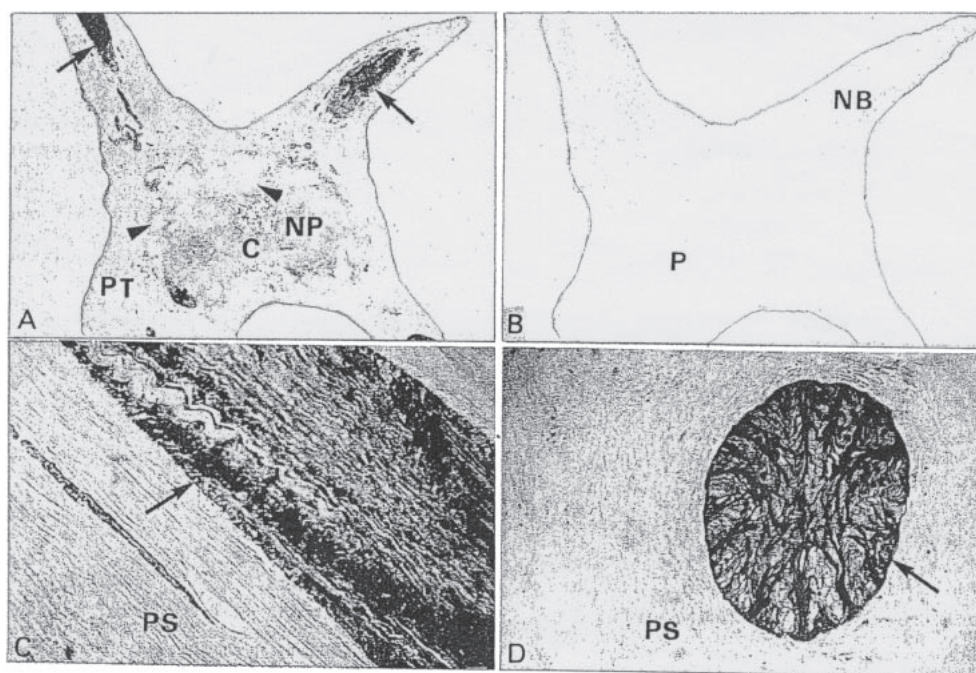


Fig. 4. Immunostaining with anti-FGL-IIb antiserum of cryostat sections of the abdominal ganglion of *Aplysia kurodai*. A: Horizontal section of the abdominal ganglion, nerve bundles (arrows) were stained. Neuron (arrowheads), neuropil (NP), commissure (C) and periganglionic tissue (PT) were scarcely stained,  $\times 23$ . B: Horizontal section stained with the antiserum after chloroform-methanol (2:1, v/v) pretreatment. NB: nerve bundles, P: parenchyma,  $\times 23$ . C: Horizontal section of the proximal portion of peripheral and connective nerves of the abdominal ganglion. Arrow: nerve bundles, PS: perineural sheath,  $\times 230$ . D: Cross section of the proximal portion of peripheral and connective nerves that extended from the abdominal ganglion. Arrow: nerve bundles, PS: perineural sheath,  $\times 230$ .

HPTLC plates (Fig. 3) and several bands of proteins on nitrocellulose sheets. The antiserum stained neuropil, neuronal cell bodies, and periganglionic tissues. Pretreatment of tissue sections with chloro-

form-methanol abolished the staining of neuropil and neuronal cell bodies indicating that the staining of neuronal elements was given by antigenic glycolipids.<sup>12)</sup>

Table 1. Effect of various glycolipids on cAMP-kinase activity

Effector	Kinase activity (pmol/min)
None	2.5 ± 0.2
SGL-II	63.0 ± 2.0
HF-SGL-II	59.0 ± 2.6
SGL-I	62.3 ± 1.0
SGL-I'	55.7 ± 3.0
FGL-V	49.1 ± 1.1
F-21	43.7 ± 1.8
Glucocerebroside	2.6 ± 0.1
cAMP	111.6 ± 3.7

Kinase activity was assayed in the presence of the indicated glycolipids at 100  $\mu$ M using 40  $\mu$ M kemptide as a substrate.

Anti SGL-I antiserum reacted with several glycolipids isolated from the skin, ganglia, and nerve fibers on HPTLC (Fig. 3), and immunostained neuropil in ganglia (unpublished data).

Anti FGL-IIb antiserum stained all glycolipids with pyruvylated Gal at their non-reducing ends on HPTLC (Fig. 3) and distinctly immunostained the nerve bundles of *Aplysia*, and staining was abolished by pretreatment of tissue sections with chloroform-methanol (Fig. 4).<sup>13)</sup>

#### 4. Glycosphingolipids activate cAMP-kinase<sup>23)</sup>

To determine the functional significance of glycolipids, we tested the effects of glycolipids on the activation of cAMP-kinase partially purified from the membrane fraction of the rat brain.

Various glycolipids such as SGL-II, HF-SGL-II, SGL-I, SGL-I', FGL-V, and F-21 could activate cAMP-kinase (Table 1). Among the glycolipids examined, SGL-II was the most potent, giving half-maximal activation at 32.2  $\mu$ M. Activation of cAMP-kinase was maximal with 250  $\mu$ M SGL-II using kemptide as a substrate. The effect of SGL-II was additive on kinase activity at submaximal concentrations of cAMP. Kinase activity activated with SGL-II was inhibited by the addition of protein kinase inhibitor peptide, a specific peptide inhibitor for cAMP-kinase. Its inhibitory pattern was similar to that for the catalytic subunits. Of the various substrates tested, glycolipid-stimulated cAMP-kinase could phosphorylate MAP2, synapsin I, and MBP, but not histone H1 or casein. The regulatory subunit strongly inhibited the activity of the purified catalytic subunit of cAMP-kinase. This inhibition was reversed by the addition of SGL-II, similar to cAMP. SGL-II was capable of partially dissociating

cAMP-kinase, as observed with gel filtration column chromatography. However, the binding activity of cAMP to the holoenzyme was not inhibited with SGL-II. These results demonstrate that glycolipids can directly activate cAMP-kinase in a manner similar, but not identical, to that of cAMP.

PKC and  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase were not activated with glycolipids, indicating that the activation of cAMP-kinase by glycolipids was fairly specific.

#### 5. Discussion

The impetus for the present research on glycosphingolipids in a mollusc, *Aplysia kurodai*, was our unexpected finding that neuronal cell bodies bulkily isolated from pig brain stems contained lower amounts of gangliosides per protein than that of the cerebral cortex of the pig brain.<sup>5)</sup> Gangliosides, which have been assumed to be indispensable for neuronal functioning in vertebrates, were not detected in the nervous tissues of *Aplysia*, but more than thirty spots were detected by thin-layer chromatography of the water-soluble glycolipid fraction of tissues of *Aplysia*.<sup>11)</sup> In this context, we will discuss the structures, tissue distributions, and biological functions of newly found glycolipids in comparison with those of gangliosides that have been thoroughly studied by many investigators.

**Chemical structures.** Nine glycosphingolipids, which are supposed to be present in the nerve tissues of *Aplysia*, are pentaosylglycosphingolipids having one to three moles of 2-aminoethylphosphonic acid and/or one mole of phosphoethanolamine bound to sugars. Their common core structure,  $\text{Gal}(\beta \text{ or } \alpha)1 \rightarrow 3\text{GalNAc}\alpha 1 \rightarrow 3\text{Gal}\beta 1 \rightarrow 4\text{Glc}\beta 1 \rightarrow 1\text{ceramide}$ , is very similar to the core structure of GM1,  $\text{Gal}\beta 1 \rightarrow 3\text{GalNAc}\beta 1 \rightarrow 4\text{Gal}\beta 1 \rightarrow 4\text{Glc}\beta 1 \rightarrow 1\text{ceramide}$ , which is abundant in the mammalian brain. The glycosphingolipids of *Aplysia* can be divided into three groups by their modified sugars, 3-*O*-MeGal, 4-*O*-MeGlcNAc, and 3,4-*O*-(*S*-1-carboxyethylidene)Gal, at their non-reducing ends. 2-Aminoethylphosphonic acid is characterized by its C-P bond and its natural occurrence was reported by Horiguchi and Kandatsu for the first time.<sup>24)</sup> The glycosphingolipids of *Aplysia* may be acidic owing to the bound 2-aminoethylphosphonic acid or phosphoethanolamine. Five sphingoglycolipids have pyruvic acid and their acidity may be comparable to monosialo- or disialogangliosides.

Recently, the structures of two major glycosphingolipids isolated from the eggs of *Aplysia* were

determined, with one being tetraosyl- and the other octaosylglycosphingolipid. Both glycolipids have three moles of aminoethylphosphonic acid and share the structure  $[6'-O-(2\text{-AEP})\text{Gal}\alpha 1\rightarrow 2] (2\text{-AEP}\rightarrow 6)\text{-Gal}\beta 1\rightarrow 4(2\text{-AEP}\rightarrow 6)\text{Glc}\beta 1\rightarrow 1\text{ceramide}$  with SGL-I.<sup>25),26)</sup>

**Tissue and cellular distribution of glycosphingolipids in nerve tissues of *Aplysia*.** Antisera against SGL-II and SGL-I, which are specific to 3-*O*-MeGal and 4-*O*-MeGlcNAc, respectively, stained neuropil of ganglia. Furthermore, anti SGL-II antiserum stained perineural tissues. Antiserum against FGL-IIb, which is specific to pyruvic acid bound to galactose at the non-reducing end of glycolipids, only stained nerve bundles distinctly. Sherbany *et al.* reported that several kinds of phosphonoglycolipids were synthesized in R2 neuronal cell bodies and were transported to the periphery in the axons of *Aplysia californica*.<sup>27)</sup> Though SGL-II sent by us as a reference to them was not identified, their glycolipid fraction may contain pyruvic acid-containing glycosphingolipids isolated from *Aplysia kurodai*.

**Neurobiological significance of glycosphingolipids isolated from *Aplysia*.** Accumulating evidence suggests that membrane protein functions are regulated by surrounding lipids in the lipid bilayer. Previous studies have reported that the activities of several protein kinases, including PKC,<sup>28)</sup>  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II,<sup>29)</sup> epidermal growth factor receptor kinase,<sup>30),31)</sup> and ganglioside-dependent protein kinases<sup>32)-34)</sup> were modified by glycolipids. Yates *et al.*,<sup>35)</sup> Chan,<sup>33)</sup> and we<sup>36)</sup> reported that the activity of the catalytic subunit of cAMP-kinase was inhibited by gangliosides. On the other hand, we found that the glycolipids could stimulate the activity of the holoenzyme and did not affect the activity of the catalytic subunit. These differences should be due to differences in chemical and stereochemical structures between SGL-II and GM1. The glycolipids used in our study were a series of compounds containing 2-aminoethylphosphonate isolated from the skin, ganglia, and nerve fibers of the marine mollusc, *Aplysia kurodai*, and did not contain sialic acids. Compounds consisted of a common oligosaccharide backbone ( $\text{GalNAc}\alpha 1\rightarrow 3\text{Gal}\beta 1\rightarrow 4\text{Glc}$ ). This structure is specific for this series of glycolipids in the animal kingdom. As for the ceramide moiety, most fatty acids were palmitate and about half of the bases were anteisonadeca-4-sphingenine in the glycolipids. Thus, the glycolipids used were dissimilar to gangliosides in their lipophilic moieties.

Though most glycolipids are bound to the membrane surface of cells, 2~5% of total cellular glycolipids are soluble in the cytosol,<sup>37)-39)</sup> and occur as micelles in aqueous solution at the concentrations used in the present study. Therefore, glycolipids can reach specific intracellular components at physiologically significant levels. The content of SGL-II was 3 mg/g of dry weight in consideration of the yield. As dry weight accounts for ~10% of wet weight tissue, the concentration of SGL-II in the total tissues of *Aplysia* may correspond to ~180  $\mu\text{M}$  in the tissues of *Aplysia*. This concentration of glycolipids is enough to activate cAMP-kinase, as shown in the present study. However, further study is still required to understand the molecular mechanism how these glycolipids activate soluble enzymes in the cytosol.

*Aplysia*'s nervous system has been used successfully to elucidate the cellular and molecular processes underlying long-term potentiation of synaptic conductance. Cyclic AMP-kinase was supposed to be involved in these processes.<sup>40)</sup> Catalytic and regulatory subunits of cAMP-kinase and their cDNA were identified in the neurons of *Aplysia*.<sup>41)</sup> Furthermore, synthesis of phosphosphingoglycolipids in neuronal cell bodies and their axonal transport were reported in *Aplysia*, as previously described.<sup>27)</sup>

We suppose that in *Aplysia* glycolipids may be involved in the long-term potentiation of synaptic conductance through activation of cAMP-kinase.

It was reported that exogenous glycolipids induced differentiation of several types of neural cells with concomitant sprouting and extension of neurites.<sup>42)</sup> In fact, we found that NG108-15 cells increased the extension of neurites in the presence of SGL-II (unpublished data). Glycolipids may be involved in many neural functions via the activation of cAMP-kinase.

Many sphingoglycolipids, other than those reported in this study, have been identified in protostomia.<sup>43)</sup> Some of them, or yet unveiled glycolipids, may share biologically or neurobiologically important functions proposed for gangliosides in vertebrates.

## 6. Conclusion

Gangliosides have never been identified in the phylum of protostomia, though the presence of polysialic acid was shown in the embryos of *Drosophila*.<sup>44)</sup> Glycosphingolipids identified in the nerve tissues of *Aplysia*, especially pyruvylated acidic glycosphingolipids, may share neurobiological functions proposed for gangliosides in vertebrates.



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## Profile

Mei Satake was born in 1929 and received M.D. in 1953 and Ph.D. (research on the structure of dermatansulfuric acid in the laboratory of Prof. Hajime Masamune) in 1959 from Tohoku University. From 1959 to 1961 he carried out his research work on the biosynthesis of protein in the brain in the laboratory of Prof. Kikuo Ogata, the Neurochemical Section of the Neurosurgical Institute, Niigata University School of Medicine. From 1961 to 1963 his work on the structural analysis of  $\alpha_1$ -acid glycoprotein was carried out in the laboratories of Prof. Karl Schmid, the Lovett Laboratory, M.G.H. and the Department of Biochemistry, Boston University. In 1963 he stayed in the Department of Histology, Gothenburg University and learned Prof. Holger Hyden's unique methods for the analysis of the single neuron freshly prepared from the brain. After coming back to Japan from Sweden he had set his research project to chemical characterization of the neuron and started to invent methods to isolate neurons in bulk from animals. His first method was reported in 1966. He was promoted to Professor of the Neurosurgical Institute, Niigata University School of Medicine in 1965. Until his retirement in 1995 from the Brain Research Institute, Niigata University he had engaged in studies on the chemical plasticity of hemispherectomized rat and nucleic acid turnover in the hibernating bat brain besides the chemical characterization of the neuron. He is an honorary member of the Japanese Society for neurochemistry and the International Society for Neurochemistry.



## Profile

Eishichi Miyamoto was born in 1936, and received M.D. in 1963 and Ph.D. in 1968. He started his research career in 1964 with studies on the isolation and identification (*N*-acetyl- $\alpha$ -aspartylglutamate) of peptides in brain under guidance of Dr. Y. Kakimoto and Prof. I. Sano at Department of Neuropsychiatry, Osaka University School of Medicine. After Graduate Course of Medicine, he joined the laboratory of Prof. P. Greengard, a winner of the Nobel Prize, at Department of Pharmacology, Yale University School of Medicine in U.S.A. and first found cAMP-dependent protein kinase in brain in the world. This discovery led to the studies on the functional role of cAMP in brain and other various tissues. After he came back to Japan, he was studying on signal transduction of a variety of the cells through activation of several protein kinases. One of them was  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II which was later found to exert functional roles in the cells by its activation with the low level of intracellular  $\text{Ca}^{2+}$ . Especially this kinase was found to be involved in development of long-term potentiation in the hippocampus of the brain, which was a model of neuronal plasticity like memory. He was promoted to Professor at Department of Pharmacology, Kumamoto University School of Medicine in 1979 and retired from the University in 2002. He was also President of the Japanese Society for Neurochemistry in 1995 to 1997, President of the Japanese Pharmacological Society in 1998 to 2000, a council member of the Asian-Pacific Society for Neurochemistry in 1996 to 2000 and a council member of the International Society for Neurochemistry in 1997 to 2001.

