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Ganglioside/protein kinase signals triggering cytoskeletal actin reorganization

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Exposure of neuronal cells to nanomolar concentrations of oligosaccharide portions of ganglioside GM2 and GT1b stimulates cAMP-dependent protein kinase (PKA) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), respectively, in a few seconds suggesting the presence of glyco-receptor-like molecules on the surface of the cells. Both GM2/PKA (GalNAc/PKA) and GT1b/CaMKII signaling cascades induced cytoskeletal actin reorganization through Cdc42 activation leading to filopodia formation within 2 min. Long-term effects of these glyco-signals were facilitation of dendritic differentiation of primary cultured hippocampal neurons and cerebellar Purkinje neurons indicating physiological roles of the signals in neuronal differentiation and maturation.
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Introduction

Cell-surface saccharide chains are involved in cell-cell interactions, and the saccharide-mediated recognitions appear to be capable of activating intracellular signal-generating systems [1]. Some saccharide chains are distributed in restricted cells. Some gangliosides, for example, are expressed at neuronal cell membranes in a specific area of the central nervous system (CNS). These suggest their potential involvement in neuronal development and function. Saccharide-chains of gangliosides are synthesized by sequential transfer of single saccharides [2]. In mice, which synthesize GM2 ganglioside, the lacking of GalNAc transferase results in a deficiency of all complex gangliosides including GD1b and GT1b. The remaining brain gangliosides are GD3 and GM3. Although GalNAcT (−/−) mice develop to reproductive age with grossly normal nervous system morphology and physiology, they display axonal and myelin degeneration in both the central and peripheral nervous systems [3] and their cerebellar granule neurons lose resistance to depolarizing conditions and undergo apoptosis *in vitro* [4]. Moreover, behavioral defects became evident as they aged [3]. Much more severe lethal defects appear in mice lacking both GM2 synthase [5,6] and GD3 synthase [7,8]. Of the double knockout mice that express GM3 as an only brain ganglioside, about 50% died by 13 weeks of age and exhibited lethal seizures by weak sound stimulation after apparently normal development up to 6 weeks [9]. These observations indicate that gangliosides are indispensable for maintenance of neural function to protect animals from some sorts of stress. However, the molecular mechanism of ganglioside-mediated regulation of neural function has not been characterized well whereas some gangliosides facilitate synaptic plasticity [10–14], and some gangliosides initiate neuritogenesis in some neuroblastoma cell lines.

One of the possible molecular mechanisms is protein kinase modulation. Ganglioside-mediated protein kinase modulation was analyzed either *in vitro* or in *in vivo* systems [1]. These studies include regulation of growth factor receptor tyrosine kinase, membrane-associated or cytosolic protein kinases, and membrane-microdomain-mediated protein kinases.

TrkA is the high-affinity tyrosine kinase-type receptor for nerve growth factor (NGF). TrkA activity is enhanced by GM1 ganglioside by direct interaction of the ganglioside and the receptor [15]. Epidermal growth factor receptor (EGFR) activity is inhibited by GM3 [1] and enhanced by GD1a [16]. The mechanism of the inhibitory effect of GM3 was recently demonstrated to be the direct interaction of the extracellular domain of EGFR and the ganglioside [17]. The mechanism of the enhancing effect needs further extensive study.

Several ganglioside-activated kinases have been demonstrated in a cell-free system since the first documentation of ganglioside/ Ca^{2+}-dependent protein kinases [18,19]. There are

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several ganglioside-dependent modulations of well-known cytosolic protein kinase activities in the cell-free system. Gangliosides suppress phospholipid and Ca^{2+}-dependent activity of protein kinase C (PKC) [20]. Contrary to this, PKC is activated by GM3 with phorbol ester as substitutes for the phospholipids [21]. PKA activity is stimulated by gangliosides [22], while cAMP-independent activity of catalytic subunit of PKA is suppressed by gangliosides [23]. CaMKII activity is modulated by gangliosides [24]; in the absence of Ca^{2+}/calmodulin, gangliosides activate CaMKII, while higher concentrations of gangliosides prevent the activity and Ca^{2+}/calmodulin-activated CaMKII is inhibited by the gangliosides. The mechanisms of this complex regulation of CaMKII activity by gangliosides are direct interactions between the gangliosides and regulatory domains of the kinase and between the gangliosides and Ca^{2+}/calmodulin [25,26]. Since gangliosides are mostly believed to be localized restrictedly in the outer leaflet of the membrane bilayer, further study is needed to assign physiological roles to these ganglioside-mediated modulations of the cytosolic protein kinases.

Since the pioneer work of Roisen *et al.* [27], there have been a number of documents of neuritogenesis of neuroblastoma cells induced by exogenously added micromolar levels of gangliosides. The probable mechanism is c-Src activation mediated by the glycosphingolipid-enriched domain (GEM) structure and function. Micromolar gangliosides are incorporated into the plasma membrane and concentrated to the microdomains, the so-called GEM, or rafts and the associated signal transducers such as c-Src are activated [28]. Details are discussed below. An ectokinase is activated by GQ1b at nanomolar levels triggering neuritogenesis of a human neuroblastoma cell line, GOTO [29]. Even in this nanomolar ganglioside reaction, the ceramide portion of GQ1b is essential to the kinase activation. In contrast to these differentiation effects, some gangliosides stimulate proliferation of the cells. Micromolar GM1 stimulates DNA synthesis and proliferation of U-1242 MG human glioma cells via activation of mitogen-activated protein kinase, Erk2 and p70 S6 kinase [30]. The mechanism of the activation needs further study.

The above ganglioside-mediated reactions are interactions between the ganglioside and the target molecule in the same membrane of the cell ("cis" interaction). There is some ganglioside-mediated intercellular interaction ("trans" interaction). Carbohydrate-carbohydrate interactions mediated by gangliosides are observed in some intercellular recognition systems [31,32]. The membrane microdomain structure and function also mediate the reaction to activate a GEM-associated protein kinase, focal adhesion kinase (FAK), and GTP-binding proteins, Ras and Rho [33]. Myelin-associated glycoprotein (MAG) inhibits nerve regeneration by binding to nerve cell surface gangliosides [34]. The inhibitory effect of MAG is overcome by PKA activation [35,36].

Here we propose another probable ganglioside-mediated intercellular recognition: cell-cell interaction mediated by glyco-receptor-like molecules wherein glyco-chains presented on a cell surface are recognized by the glyco-receptor-like molecule of the recipient cells. We found that the glyco-signals received by probable glyco-receptors induced cytosolic protein kinase activation leading to cytoskeletal actin reorganization and dendritogenesis. Two isolated but similar saccharide-signaling pathways regulate cytoskeletal actin reorganization. GT1b and GM2 are the biologically active cell surface glycoconjugates. They are located at synaptic areas (see below). Nanomolar levels of oligosaccharides or gangliosides activate intracellular protein kinases followed by polymerization of cytoskeletal actins to form filopodia in neuronal cells immediately after exposure of the cells to the glycoconjugates. Furthermore, long-term exposure of primary cultured CNS neurons to the same glycoconjugates accelerates dendritogenesis. Two similar but isolated saccharide-signaling pathways conduct the reaction: one is the GT1b/CaMKII signal and the other is the GalNAc/PKA signal. In this review, we describe overviews of these two saccharide signals and their possible roles in neuronal development and maintenance of neural functions.

GT1b/CaMKII signal

Short-term effects of GT1b/CaMKII signal

Immunohistochemical examinations provided evidence that GT1b ganglioside is markedly expressed in synapses in the brain [37]. We have examined the ability of the gangliosides to signal a neuronal response. We focused on the question if intracellular CaMKII activity is modulated by gangliosides, since CaMKII is related to various neuronal functions such as neural plasticity, and may have an important role in stabilizing the dendritic architecture [38]. We employed a novel imaging technique using a fluorescence-labeled peptide substrate AS2 to measure the activity of CaMKII [39] and demonstrated that the local activation of CaMKII occurred as a result of exposure of neural cells to GD3, GD1b, GT1b, and its oligosaccharide portions (Figure 1; see Figure 4C for the method for microscopic monitoring of a fluorescence probe in living cells). When neuronal cells such as primary cultured hippocampal neurons and neuroblastoma cells are exposed to GD3, GT1b, GD1b, or

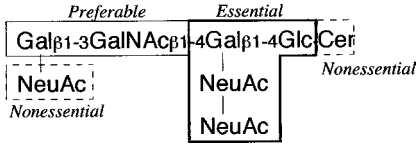

Figure 1. Oligosaccharide structure active for GT1b/CaMKII signal. Disialosyl lactose structure is a minimal essential structure for GT1b/CaMKII signal activation. GD1b structure is preferable being active at one-order lower concentrations than GD3. A terminal sialosyl residue of GT1b is nonessential and noninhibitory to the activity. Additional sialosyl residue of GQ1b prevents the activity. Ceramide portion of the ganglioside are nonessential.

GT1b oligosaccharide at 0.5–10 nM, cortical CaMKII is activated in seconds and the reaction is reversed by removal of the saccharides [40,41]. The quick and reversible nature suggests a glyco-receptor-mediated reaction.

This stimulus involved the actin-mediated filopodia formation (Figure 2B) via activation of Cdc42, a member of Rho family GTPases, which promotes filopodia formation [42].

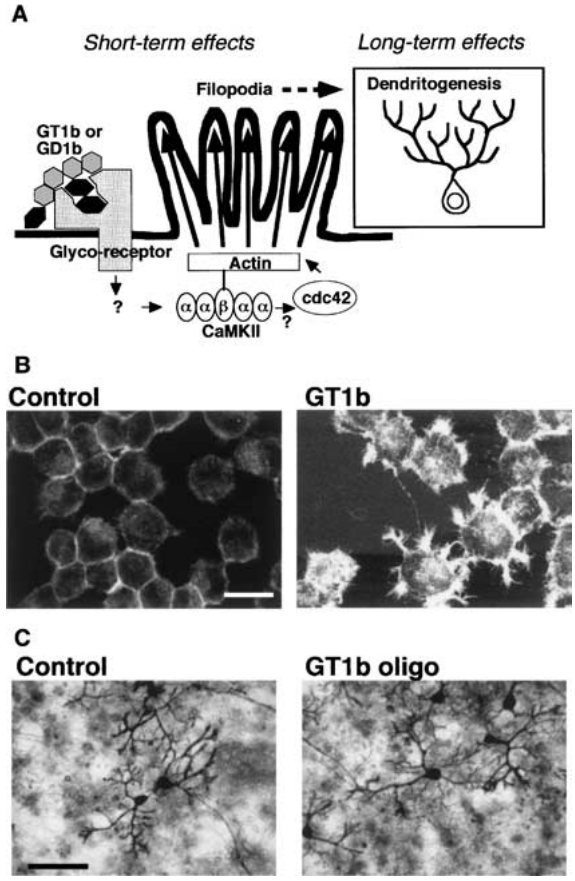

Figure 2. (A) GT1b/CaMKII signaling cascade. Oligosaccharide portions of GD1b or GT1b ganglioside are preferably recognized by probable cell-surface glyco-receptor and the saccharide signal is transduced to an intracellular signal to activate CaMKII in seconds. The saccharide activates local CaMKII that is presumably anchored to actin via CaMKIIβ isoform. Activated CaMKII induces cytoskeletal actin-reorganization to form filopodia via Cdc42 activation within 2 min. Long-term exposure (3–7 days) of the cell to the saccharide enhances dendritogenesis of primary cultured neurons. Exposure to oligosaccharides probably mimics intercellular recognition via saccharide-receptor interaction. (B) Neuroblastoma-glioma hybridoma cells (NG108-15 cells) were either untreated (control) or exposed to GT1b (approx. 4.7 nM) for 2 min at 37°C. The cells were then fixed and stained with rhodamine-phalloidin and observed with a confocal fluorescence microscope. Scale bar, 10 μm. (C) Primary cerebellar neurons were either untreated (control) or exposed to 6.3 nM of GT1b oligosaccharide (GT1b oligo) for 7 days from days *in vitro* (DIV) 8 to 14. Cells were fixed and stained with anti-calbindin D-28K to visualize Purkinje cells. Scale bar, 100 μm.

The efficiency of the saccharides are GT1b $\approx$ GD1b $\approx$ GT1b oligosaccharide > GD3 (Figure 1). An essential saccharide structure may be the oligosaccharide portion of GD3 ganglioside, but GD1b and GT1b are effective at one-order lower concentrations than GD3. External Gal-GalNAc residue of GD1b and GT1b was preferable for the activity. External sialyl residue of GT1b did not prevent the activity, but additional sialyl residue of GQ1b prevented the reaction. Ceramide portions of the gangliosides are not essential and GT1b oligosaccharide is effective at the same concentrations as the parental ganglioside.

Actin polymerization and filopodia formation are obvious in 2 min. These rapid reactions strongly suggest that in particular in neural cells, oligosaccharide portions of the gangliosides could be recognized by an intracellular signal-generating system via a probable cell-surface glyco-receptor, and such signals might be capable of regulating some aspects of neural plasticity and dendritic architecture, through the modulation of intracellular protein kinase activities (Figure 2A).

Long-term effects of GT1b/CaMKII signal

We then examined the long-term effects of the GT1b/CaMKII signaling on dendritic formation since besides filopodia formation, Cdc42 is related to the initiation of dendritogenesis [43–45]. GT1b oligosaccharide facilitated elongation and branch formation of dendrites of hippocampal neuron and cerebellar Purkinje neurons grown *in vitro*, when the cells were exposed to the saccharide for 3–7 days (Figure 2).

When cultured rat hippocampal cells were exposed to 4.7 nM GT1b, actin polymerization was promoted in neurons within 2 min; the reaction is neuron specific and non-neuronal cells in the culture did not react to GT1b. Exposure of the same neurons to 10 nM GT1b oligosaccharide for 3 days drastically enhanced actin-rich dendrite generation. The hydrophobic ceramide portions were not necessary for the reaction, indicating that membrane incorporation of the glycoconjugate is not the mechanism of the reaction.

Cerebellar Purkinje neurons can develop characteristic dendrites as grown *in vivo* under the *in vitro* culture conditions of Furuya *et al*. [46], in which cerebellar granule neurons make synapses on dendrites of Purkinje neurons [47] (Figure 3). In mixed cerebellar cell culture, Purkinje neurons develop dendrites and the development was accelerated when the cells were exposed to 6.3 nM GT1b oligosaccharide for 7 days (Figure 2C).

Physiological aspects of GT1b/CaMKII signal

GD1b is distributed in granule cell surface in rat primary cerebellar culture as well as in brain [48] (Figure 3). GT1b is distributed in the molecular and the granular layers [37]. GD1b is localized in all layers of cerebellar cortex at early postnatal days [49]. These findings indicate that gangliosides are localized in the synaptic area as well as in the cell body and axons of

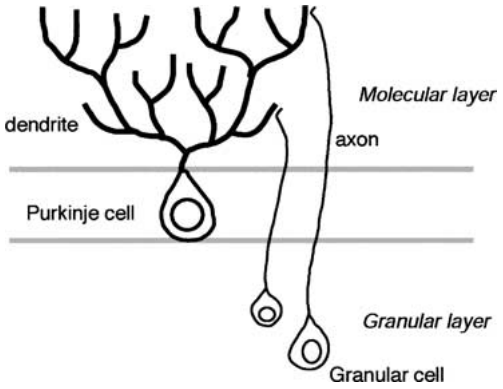

Figure 3. Synapse formation between cerebellar granular cell and Purkinje neuron. Purkinje neurons elongate their dendrites to molecular layer of cerebellum. Granular layer consists of granular cells (neurons) which elongate axon to the molecular layer to make synapse with dendrites of Purkinje neurons. Immunohistological study showed granular cells contain GT1b and GD1b and the gangliosides are present in molecular layer, thus their axons most probably express these gangliosides.

cerebellar granular cells. While these gangliosides are not detectable in Purkinje neurons, the gangliosides are present at molecular layer [37] (*i.e.,* axons of the granular cells) where dendrites of Purkinje neurons grow into (Figure 3). Thus cell-cell interaction between Purkinje neurons and other neurons could be mediated by the gangliosides, and GT1b/CaMKII signaling may play roles in the physiological dendritic reaction resulting in synapse formation.

GT1b/CaMKII signaling seems to be a local reaction and is dependent on transient increment of intracellular Ca^{2+} + levels by the Ca^{2+} + release from the intracellular stores [41], whereas micromolar gangliosides with ceramide portion induce Ca^{2+} influx in some cells [50]. Furthermore, the depletion of Ca^{2+} from extracellular media did not prevent the reaction. Such a local reaction is probably mediated by anchored enzymes.

CaMKII plays a major role in neuronal plasticity, such as spatial learning and long-term potentiation in the hippocampus [51–53], and is involved in signal transduction in various cell reactions. CaMKII may also have important roles in stabilizing the dendritic architecture [38]. It is interesting to note that CaMKII is targeted to the dendritic cytoskeleton and actin via CaMKIIβ isoform [54]. CaMKII is present as holoenzyme consisting of a number of molecules of a mixture of alpha and beta isoforms in neuron. While the alpha isoform is the major component of neurons and a cytosolic enzyme by itself, the beta isoform binds to actin cytoskeleton and docks a large fraction of alpha isozyme to synaptic and cell cortex sites of actin as targeting module by forming the heterooligomers (Figure 2A). Actin is highly enriched in dendritic spines and cell cortex [55,56]. The anchoring to actin filaments may not only target them to dendritic spines but also trigger actin reorganization by the activated kinases. The actin targeting of these kinases should be beneficial for mediating actin polymerization.

We showed that the gangliosides stimulated Cdc42 via CaMKII [41]. At present, the mechanism of activation of Cdc42 by CaMKII is unknown. However, we found that *N*-methyl-D-aspartate (NMDA) stimulated similar actin reorganization via Cdc42 activation. In addition, NMDA induced faint activation of Rac1. While GT1b induced calmodulin independent CaMKII activation, NMDA induced Ca^{2+}/calmodulin-mediated CaMKII activation since the reaction is blocked by the inhibitor of calmodulin. Besides Cdc42 activation, the Ca^{2+}/calmodulin activation should stimulate the signal pathway of Rac1 activation, and the gangliosides did not activate this pathway.

Behavioral deficits and neuronal degeneration displayed by ganglioside-deficient mutant adult mice [3,9] demonstrate that gangliosides are indispensable for maintenance of neural function to protect animals from exogenous stresses. In our experiments, GT1b and GD1b were potent activators followed by GD3 for CaMKII activation and filopodia formation. The former two gangliosides were effective at least at 10 ng/ml (4.7–5.4 nM), while at least 100 ng/ml (70 nM) of the latter one was necessary to stimulate clear filopodia formation. It is interesting to note that this potency seems to correlate well with the phenotypic severity of the mutant mice [9]. GD3 may compensate the functions of the other complex gangliosides but incompletely or less potently than GD1b and GT1b.

In cell-free systems, certain gangliosides activate CaMKII [24,26] with GT1b being the most superior activator. However, the effective concentration is much higher than in the GT1b/CaMKII signaling system, and the oligosaccharides in the extracellular media are most unlikely to rapidly incorporate into the cells. Furthermore, activation by the oligosaccharide and the streptavidin-BSA-based neoglycoprotein suggests the involvement of cell-surface receptors but not membrane incorporation of the glycoconjugates. Moreover, the effects of these non-lipid compounds disappear immediately after they are washed out. Thus, the involvement of a cell surface glycoreceptor is the most probable.

GalNAc/PKA signal

Short-term effects of GalNAc/PKA signal

GM2 ganglioside, which contains a GalNAc-residue at its non-reducing terminal, seems to be an attractive candidate for another biologically active cell surface glycoconjugate. GM2 is markedly expressed in cortical and other neurons during the period of normal dendritogenesis in various mammals, and that elevated GM2 expression is correlated with ectopic dendritogenesis of cortical neurons in patients with human neuronal glycosphingolipid storage diseases like GM2 gangliosidosis and Niemann-Pick disease type C [57–60]. The elevation of GM2 has thus been considered to play a role in normal and ectopic dendritogenesis. However, the molecular mechanism of the event has not been well characterized.

In addition to CaMKII, we wondered if intracellular PKA activity is modulated by gangliosides, since PKA is also related to various neuronal functions such as spatial learning, long-term potentiation in the hippocampus [61–63], neurite outgrowth [64] and regeneration [35,36] as well as is involved in signal transduction in various cell reactions.

We employed a novel imaging technique to measure the activity of PKA [65], and showed that the local activation of PKA occurred as a result of exposure of neural cells to saccharides containing GalNAc residue at the nonreducing termini (GalNAc-S) [66,67] as shown in Figure 4.

This stimulus involved the actin-mediated filopodia formation in neuronal cells via activation of Cdc42 (Figure 5A and B) [67,68].

GalNAc-terminated oligosaccharides and glycoconjugates such as GM2, GA2, Gb5, GM2 oligosaccharides, and GalNAcα1-4 oligomers are almost equally active at 5–200 nM. Both of the oligosaccharides containing alpha and beta anomers of the non-reducing terminal GalNAc residue are active (Figure 6). GD2 and Gb4 are inactive perhaps because of the spatial hindrance of the terminal GalNAc residue by neighboring saccharide residues. GM2 ganglioside is a most probable candidate of a natural ligand in this signaling. Since the disaccharide GalNAcα1-4GalNAc cannot activate the reaction, at least 3–4 saccharide residues, such as GM2 oligosaccharide, are essential for activation.

Exposure of neuronal cells such as primary cultured hippocampal neuronal cells to GalNAc-terminated glycoconjugates promotes PKA activation via adenylate cyclase activation.

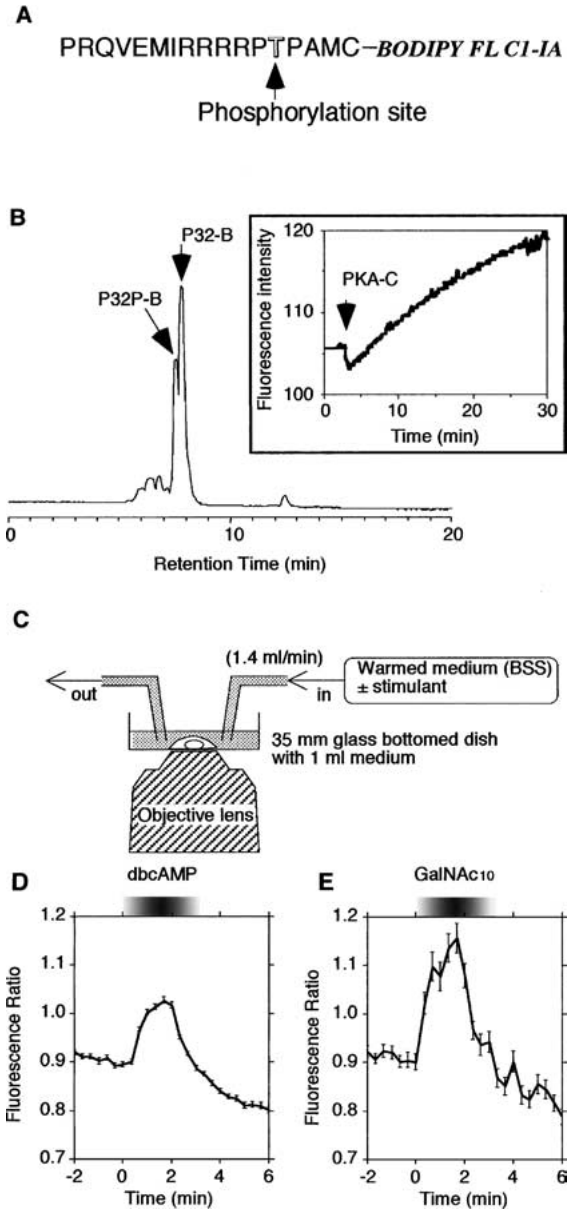

Figure 4. Novel fluorescent peptide substrate, P32-B for imaging of PKA activity in living cells. (A) P32-B was constructed from a peptide consisting of 17 amino acids of the PKA phosphorylation site of Mr 32,000 dopamine- and cAMP-regulated phosphoprotein (DAPP32), conjugated with the fluorescence probe, BODIPY FL C1-IA (Molecular Probe, Eugene, OR) at its C-terminus Cys residue. P32-B is more resistant than ARII or DRII [65] to cytosolic proteases. (B) *In vitro* phosphorylation of P32-B. P32-B of 0.2 μg/ml dissolved in aqueous solution consisting of 50 mM Hepes (pH 7.5), 10 mM $MgAc_2$, 1 mM EGTA, and 0.5 mM ATP was kept at 30°C and fluorescence at 510 nm exited by 495 nm light was monitored (B, inset). Catalytic subunit of PKA (PKA-C) was added to final concentration of 2 μg/ml at the time indicated. The fluorescence of P32-B is increased 50% by complete phosphorylation. An aliquot of the sample taken after 24 min of PKA-C addition was analyzed by HPLC using C18 column and 0.1% TFA aqueous solvent with acetonitrile gradient [65]. (C) Monitoring of P32-B phosphorylation in living NG108-15 neuroblastoma-glioma hybrid cells. The cells cultured on poly-lysine-coated glass bottomed dish (35 mm) were loaded with the substrate by incubation with 6 μg/ml of P32-B in balanced salt solution (BSS), pH 7.3, consisting of 130 mM NaCl, 5.4 mM KCl, 20 mM HEPES, 5.5 mM glucose, 0.8 mM $MgSO_4$, and 1.8 mM $CaCl_2$ for 30 min at 25°C. The substrate-loaded cells were conditioned by passage of BSS (1.4 ml/min) at 30°C, then exposed to the stimulating reagent; 1 mM dibutyryl cAMP (dbcAMP) in (D) or 49 nM GalNAc(α1-4) decamer in (E) by adding to the BSS passage; the fluorescence of the stained cells was then measured using 480 nm excitation and subjected to image analysis, using an image processor (Argus 100 or Argus 50, Hamamatsu Photonics, Shizuoka, Japan). After 2 min of exposure, the media was restored to BSS without the reagent. The extracellular media in the assay dish was maintained at 1 ml. Thus, by this bath application method, the concentrations of applied saccharides reach maximum at about 40 s after the application starts and almost completely washed out at about 40 s after the end of the application. The time course of the averaged fluorescence ratios in cytosolic areas of 28 (D) and 21 cells (E) were plotted every 20 s. The ratio was calculated by dividing the fluorescence intensity at every 20 s by that at time −4 min. Error bars show standard error.

Intracellular Ca^{2+} levels are not changed, and depletion of extracellular Ca^{2+} did not affect the reaction.

PKA activation is obvious in seconds and actin polymerization and filopodia formation are obvious in 2 min. Thus, in particular in neural cells, oligosaccharide portions of the gangliosides can be recognized by a glyco-receptor-like molecule on the neuronal cell surface, which generates intracellular signals to regulating some aspects of neural functions through the modulation of intracellular protein kinase activities.

Long-term effects of GalNAc/PKA signal

Since Cdc42 is related to the initiation of dendritogenesis [43–45] in addition to filopodia formation, we then examined long-term effects of the GalNAc/PKA signaling on dendritic formation. This signaling system may have a physiological relevance to dendritic growth of neurons because 3–6 days exposure to GalNAc-terminated oligosaccharide facilitated elongation and branch formation of dendrites of hippocampal neurons and cerebellar Purkinje neurons grown *in vitro* (Figure 5).

When cultured rat hippocampal cells were exposed to 49 nM GalNAc(α1-4) decamer ($GalNAc_{10}$), actin polymerization was promoted in neurons within 2 min and the reaction is neuron specific, and non-neuronal cells in the culture did not react to the saccharide. Exposure of the same neurons to 100 nM GM2 oligosaccharide for 3 days drastically enhanced F-actin-rich dendrite generation (Figure 5C). The hydrophobic ceramide portions were not necessary for the reaction indicating membrane incorporation of the glycoconjugate is not the mechanism of the reaction.

In mixed cerebellar cell culture, Purkinje neurons develop dendrites and the development was accelerated when the cells were exposed to nanomolar levels of $GalNAc_{10}$ for 4–6 days. The positive effect of the saccharide was evident at 1 nM.

Physiological aspects of GalNAc/PKA signal

As described above, GM2 is distributed in the area of physiological and pathological dendritogenesis in the brain. Besides, GalNAc-S such as GM2 ganglioside are distributed in synaptic areas such as neuro-muscular junctions and retinal arbors [69–71]. PKA plays a major role in neuronal plasticity [51]. The GalNAc/PKA signal may play a role in such synaptic activities.

GalNAc/PKA signaling is a local reaction. PKA is anchored to other proteins by various A-kinase anchoring proteins (AKAPs). AKAPs dock PKA to selected proteins to control the

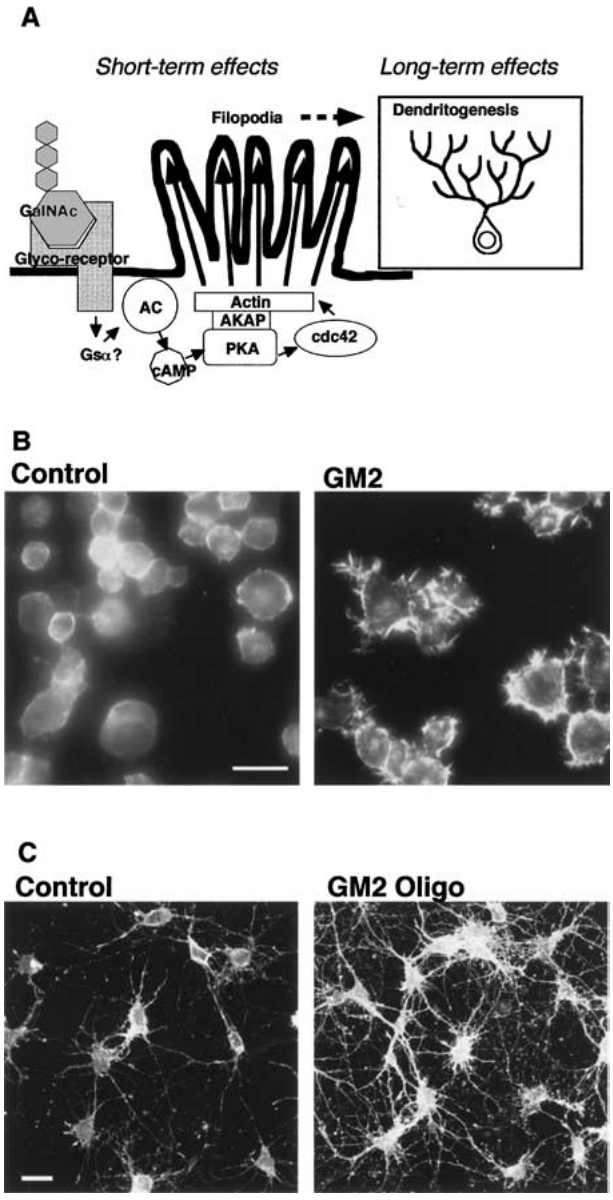

Figure 5. (A) GalNAc/PKA signaling cascade. Oligosaccharides with non-reducing terminal GalNAc residue such as that of GM2 ganglioside are probably recognized by a cell surface glyco-receptor and the saccharide signal is transduced to intracellular signal to activate local adenylate cyclase to produce local cAMP, which activates neighboring PKA in seconds. The saccharide signal presumably activates actin-anchored PKA via an A-kinase anchoring protein (AKAP). PKA activates Cdc42 in non-phosphorylation manner [87] to induce re-organization of cytoskeletal actin to form filopodia within 2 min. Long-term exposure (3–7 days) of the cell to the saccharide enhances dendritogenesis of primary cultured neurons. Exposure to oligosaccharides probably mimics intercellular recognition via saccharide-receptor interaction. (B) NG108-15 cells were either untreated (control) or exposed to extracellular media containing GM2 (approx. 72 nM) for 2 min. The cells were then fixed and stained with rhodamine-phalloidin and observed with a fluorescence microscope. Scale bar, 10 μm. (C) Rat hippocampal neurons were selectively cultured after treatment with cytosine 1-β-D-arabinofuranoside. The cultured neuron on DIV 4 were either untreated (control) or exposed to 100 nM GM2 oligosaccharide in culture media for 3 days (GM2 Oligo). At DIV 7, the cells were fixed and actin filament was stained with rhodamine-phalloidin. Confocal microscopic images of the actin filaments are shown. Scale bar, 10 μm.

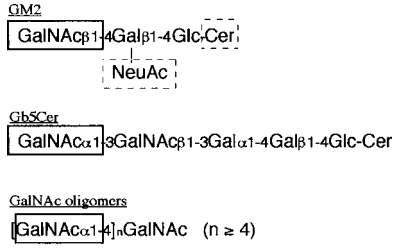

Figure 6. Oligosaccharide structure active for GalNAc/PKA signal. A GalNAc residue at non-reducing termini of saccharides is essential for GalNAc/PKA signal activation. GM2, GA2, Gb5Cer, and GalNAc(α1-4) oligomers, which are derived from a fungus *Paecilomyces* sp. I-1 [88], are active. Neither alpha nor beta configurations of the GalNAc residue are distinguished. The sialyl residue of GM2 is nonessential and non-inhibitory. The additional sialyl residue of GD2 prevents the activity. Ceramide portions of the glycolipids are nonessential. The GalNAc pentamer, but not the dimer, is active. GM2 oligosaccharide is active. Thus, at least oligosaccharides consisting of 3–4 residues are essential for the activation.

PKA reaction at restricted areas in cells. We demonstrated that PKA-anchoring is essential for a GalNAc signal using St-Ht31 inhibitor peptide, which inhibits the interaction between PKA regulatory subunit II and A-kinase anchoring protein (AKAP) [72]. Thus, GalNAc treatment activates PKA within a limited local area of the cells by adenylate cyclase activation in a limited area, *i.e.,* cell cortex. It is interesting to note that PKA is targeted to the dendritic cytoskeleton and actin via A kinase anchor proteins [73,74]. Anchoring to actin is convenient to reorganize actin for PKA.

Arakane *et al.* [22] reported that gangliosides activate PKA in a cell-free system but the effective concentrations are much higher than our GalNAc/PKA signaling system. Besides, the saccharide structure specificity differs from ours, and the oligosaccharides in the extracellular media are most unlikely to rapidly incorporate into the cells. Mice lacking GalNAc transferase (GalNAcT), an enzyme catalyzing the synthesis of GM2 ganglioside, are devoid of all complex brain gangliosides. Cerebellar granule neurons of GalNAcT-deficient mice lose resistance to the depolarizing condition and undergo apoptosis *in vitro* [4]. Although we cannot rule out the possible indirect effects of GalNAc-S on Purkinje neurons via potentiation of the granule cells, GalNAc-S did not change intracellular Ca^{2+} levels, but activated the cAMP cascade. PKA-mediated signaling can be triggered by extracellular GalNAc-terminated glycoconjugates including GM2 ganglioside in neuronal cells.

Comparison of ganglioside mediated signal transductions

Since the pioneer work of Roisen *et al.* [27], neuritogenesis by gangliosides of a number of neuroblastomas has been demonstrated by many laboratories. In all of these studies, except the work of Tsuji *et al.* [75] where nanomolar levels of gangliosides were effective, micromolar concentrations of gangliosides were necessary for the reactions. The morphological changes were usually visible after 24–48 h exposure [76]. Prinetti *et al.* [28] reported mediation by c-Src in this neuritogenesis. However, in contrast to our system, they used micromolar concentrations of gangliosides and the oligosaccharide portion alone was not effective in their system. Furthermore, gangliosides should be incorporated into the plasma membranes of the cells, which might modify artificially the properties of the glycolipid-enriched membrane domain, the so-called raft [77,78] or the glycosphingolipid-enriched microdomain, GEM [79], and activate Src family kinases associated with it. It is probable that micromolar exogenous gangliosides induce reorganization of the membrane microdomain by insertion into the plasma membrane because gangliosides with larger saccharide residues have a smaller radius of curvature. Thus, physicochemical effects of the incorporated lipids are probable. Moreover, the filopodia formation in our case is rapid, and we were unable to detect c-Src activation (N. Chen and H. Higashi; unpublished observation). Thus, the signal transduction cascades used in our studies and others are most likely different.

GT1b/CaMKII and GalNAc/PKA signals resulted in actin reorganization and filopodia formation within 2 min. Thus a glyco-receptor at the cell surface may mediate the reaction and the glyco-receptors most probably mediate intercellular recognition. The characterization of the receptors is under way.

GT1b, GD1b, or GD3 do not activate the PKA cascade, and GalNAc-S does not activate the CaMKII cascade. Different glyco-receptors probably mediate the individual signaling cascades. However, the filopodia and dendritic architectures resulting through either GT1b/CaMKII or GalNAc/PKA signaling are indistinguishable. Spatiotemporal expression of the signaling cascades may vary in brain developing and aging.

Actin polymerization and filopodia formation are closely related to synaptic activities such as spine and synapse formation, and plasticity [80–85]. In cell-cell recognition, a cell may recognize saccharides on another cell, and this may result in the reorganization of actin filaments to facilitate dendritic activities by raising the basal activity of the components for the signal transduction. Glycoconjugates, including gangliosides, are transported to neuronal termini by axonal transport [86]. Such termini may come into contact with receptors of a targeting cell to trigger saccharide signal transduction in the cell to generate dendritic activities.

These signal systems may play a role in phenotypic development, particularly dendritogenesis and successive synapse formation in developing neurons.

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