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Gangliosides stimulate bradykinin B2 receptors to promote calmodulin kinase II-mediated neuronal differentiation

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Gangliosides mediate neuronal differentiation and maturation and are indispensable for the maintenance of brain function and survival. As part of our ongoing efforts to understand signaling pathways related to ganglioside function, we recently demonstrated that neuronal cells react to exogenous gangliosides GT1b and GD1b. Both of these gangliosides are enriched in the synapse-forming area of the brain and induce Ca^{2+} release from intracellular stores, activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and activation of cdc42 to promote reorganization of cytoskeletal actin and dendritic differentiation. Here, we show that bradykinin B2 receptors transduce these reactions as a mediator for ganglioside glycan signals. The B2 antagonist Hoe140 inhibited ganglioside-induced CaMKII activation, actin reorganization and early development of axon- and dendrite-like processes of primary cultured hippocampal neurons. Furthermore, we confirmed by yeast reporter assay that major b-series gangliosides, GT1b, GD1b and GD3, stimulated B2 bradykinin receptors. We hypothesize that this B2 receptor-mediated ganglioside signal transduction pathway is one mechanism that modulates neuronal differentiation and maturation.

Keywords: bradykinin receptor/Ca^{2+} signal/cdc42/ganglioside/neuritogenesis.

Abbreviations: AM, acetoxymethyl ester; AS2, acrylodan-CPLARTLSVAGLPGKK; ATII, angiotensin II; AT1a, angiotensin II receptor 1a; B1R, bradykinin B1 receptor; B2R, bradykinin B2 receptor; BSS, balanced salt solution; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; CNS, central nervous system; DIV, days *in vitro*; GD1a, Neu5Acα3Galβ3GalNAcβ4(Neu5Acα3)Galβ4GlcCer; GD1b, Galβ3GalNAcβ4(Neu5Acα8Neu5Acα3) Galβ4GlcCer; GD3, Neu5Acα8Neu5Acα3Galβ4Glc Cer; GM1, Galβ3GalNAcβ4(Neu5Acα3)Galβ4Glc Cer; GM2, GalNAcβ4(Neu5Acα3)Galβ4GlcCer; GM3, Neu5Acα3Galβ4GlcCer; GPCR, G-protein-coupled receptor; Hoe140, D-arginyl-[Hyp[3], Thi[5], D-Tic[7], Oic[8]]-bradykinin; GT1b, Neu5Acα3Galβ3 GalNAcβ4(Neu5Acα8Neu5Acα3)Galβ4GlcCer; Oic, (3aS,7aS)-octahydroindolyl-2-carboxylic acid; PBS, phosphate-buffered saline; Tic, 1,2,3,4-tetrahydr oisoquinoline-3-carboxylic acid; Thi, L-thienylalanine.

Glycans of glycolipids, glycoproteins and proteoglycans are all exposed to cell surfaces and contribute to intercellular recognition. Gangliosides are acidic glycolipids that are major components of glycoconjugates in the brain. We found that exposure of neural cells to nanomolar levels of gangliosides GD1b and GT1b stimulates several reactions: Ca^{2+} release from intracellular stores, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) activation, cdc42-mediated cytoskeletal actin reorganization and dendritic differentiation (*1*). Mice lacking ganglioside synthesis die soon after birth, with degradation of their central nervous system (CNS) (*2*). The ganglioside signal transduction pathway that we identified is purported to mediate CNS differentiation and maintenance. The cell surface portion of the glyco-receptor has been suggested to recognize glycans, because cdc42 activation is rapid and the oligosaccharide portion of GT1b ganglioside is active as the ganglioside itself (*1*). Moreover, because ganglioside-induced release of Ca^{2+} may be mediated through phospholipase C activation via Gαq protein, trimeric G proteins are suggested to contribute to this reaction.

The present study sought to identify the molecule that transduces cell-surface glyco-signals from the extracellular ganglioside glycan into intracellular signals. As bradykinin is one of the known ligands for a G-protein-coupled receptor (GPCR) that activates cdc42 (*3–5*), we used bradykinin antagonists to determine whether bradykinin receptors may be involved in glycan-related signal transduction. We found that a bradykinin B2 antagonist did indeed inhibit glyco-signals. The gangliosides GD1b and GT1b stimulated both axon- and dendrite-like neuritogenesis in primary cultured hippocampal neurons. In addition, the B2 antagonist Hoe140 inhibited part of

the stimulating effects of the gangliosides. Using a reporter assay system constructed in budding yeast, which has a far simpler GPCR system than mammalian cells, we confirmed that the B2 bradykinin receptor (B2R) acted as a cell surface glyco-signal mediator or transducer. We propose that glyco-signals serve as a mechanism for regulating neuronal development and differentiation.

Materials and Methods

Cell culture

NG108-15 neuroblastoma-glioma hybridoma cells were cultured as described previously (*1*). Primary cultures of rat hippocampal cells were prepared from 19-day-old embryonic rat brains and cultured as described previously (*6*, *7*). Cells were plated on glass-bottom dishes at low density (8,000–10,000 cells/cm^2) and cultivated in a N2 medium as described by Clement *et al*. (*7*). The cultures consisted of a minimal Eagle's medium supplemented with N2 mixture, 0.1 mM pyruvate, 0.1% (w/v) ovalbumin and 0.01% (w/v) apo-transferrin. The B2 antagonist Hoe140 was added to the culture media at the time of plating. The cultures were then kept in a humidified atmosphere of 5% CO_2 at 37°C.

Cell culturing onto ganglioside-coated glass culture plates and neurite staining

Preparation of the culture substrates and staining of the neurites were performed as described by Clement *et al*. (*7*). Glass-bottom dishes (35 mm; Asahi Glass Co., Tokyo, Japan) were pre-coated with 15 μg/ml poly-DL-ornithine in 0.1 M borate buffer (pH 8.1) for 1 h, washed three times with H_2O and coated with 1 μg/ml ganglioside in phosphate-buffered saline (PBS) overnight at 37°C. Afterwards, the culture dishes were washed three times with PBS and flooded with N2 medium containing cells. The cells cultured were fixed, permeabilized and stained with a tubulin monoclonal antibody. The amount of ganglioside coated onto the glass culture dishes was determined by measuring the amount of sialic acids released from the ganglioside immobilized on the glass surface by heating at 80°C in 0.1 N H_2SO_4 for 1 h. The released sialic acids were quantified by fluorometric thiobarbituric acid assay (*8*).

Imaging of CaMKII activity in cells

CaMKII activity in the living cells was monitored by fluorescence imaging as described previously (*9*) using a fluorescent-labeled peptide substrate of CaMKII, acrylodan-CPLARTLSVAGLPGKK (AS2). A balanced salt solution (BSS, pH 7.3) consisting of 130 mM NaCl, 5.4 mM KCl, 20 mM HEPES, 5.5 mM glucose, 1.8 mM $CaCl_2$ and 0.8 mM $MgSO_4$ was used as the extracellular media. Calpeptin (1 μM) was added to protect the peptide substrate. Cells were cultured on poly-lysine-coated glass-bottom dishes (35 mm; MatTek, MA, USA). The cells were loaded with the substrate through incubation with 150 μM AS2 in BSS for 30 min at 25°C. The substrate-loaded cells were exposed to ganglioside and antagonist via a bath application method as described (*1*, *10*). Cell fluorescence was measured at 360 nm excitation and then subjected to image analysis using an image processor (Argus 50, Hamamatsu Photonics, Shizuoka, Japan).

Ca^{2+} imaging

Ca^{2+} indicators (Fura-2) were loaded into the cells as their acetoxymethyl ester (AM) derivatives. A stock solution of 1 mM Fura-2-AM (Dojindo Labs, Kumamoto, Japan) in DMSO was diluted 1000- or 200-fold into BSS and added to the cells cultured on the poly-lysine-coated glass-bottom dishes. The cells were then incubated at 37°C for 30 min to load and sensitize the Ca^{2+} probe by cleaving its AM group with cytosolic esterase. Images were acquired at emission wavelengths between 490 and 530 nm while exciting sequentially at 340 and 380 nm (exposure time: 1–2 s). The images were subjected to image analysis using an image processor (Aqua-C Imaging, Hamamatsu Photonics) connected to a cooled CCD camera (Orca-ER, Hamamatsu Photonics). Cells were exposed to stimulants by bath application as described previously (*1*, *10*).

Detection of polymerized actin

Cultured cells were washed three times with BSS, exposed to a stimulator in BSS with or without inhibitors for 2 min and then fixed with 4% paraformaldehyde/100 mM phosphate buffer (pH 7.4) for 15 min at 25°C. The polymerized cytoskeletal actin was visualized with rhodamine–phalloidin using the method of Ono *et al*. (*11*) with modifications (*1*).

Yeast strain, manipulations and Gα expression and Ste2p deletion constructs

Saccharomyces cerevisiae strain YKG005 (*MATa ura3-52 lys2-801 ade2-101 his3-200 leu2-3 sst2Δ1 far1Δ1 ste2Δ1 gpa1Δ3::GPA1-Gαq-c5 pep4Δ2 trp1-901 fus1::FUS1-HIS3 can1Δ::FUS1-CAN1 FUS2::FUS2-lacZ*) was derived from YPH499 (*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his1-Δ200 leu2-Δ1*) through a series of one-step gene replacements (*12*). The *FUS1–HIS3* reporter gene was integrated at the *FUS1* locus. The *FUS2-lacZ* reporter gene was integrated for the β-galactosidase reporter assay. A yeast/mammalian G protein αq-subunit chimera, GPA1-Gq-c5, was constructed by replacing the C-terminal five amino acids of Gpa1p with those of a mammalian Gαq protein (*13*).

Plasmid for yeast transformation

Plasmid pYSF1 was derived from pYES2 (Invitrogen) by replacing the *GAL1* promoter and the T7 promoter primer sites with a *STE2* promoter (0.42 kb), α factor leader (0.26 kb), FLAG tag and a polylinker (EcoRI, XhoI, SmaI, BglII, BanIII, BamHI, ApaI, SalI and SacII) at the position between the SpeI and NotI sites of pYES2. To express human B2R, we created plasmid pYSF-B2R by inserting the B2R open reading frame into the EcoRI site of pYSF1. To express murine B2R, we created plasmid pYSF-B2R by inserting the B2R open reading frame between the EcoRI

and BamHI sites of pYSF1. To express human angiotensin II receptor 1a (AT1a) receptor, we created plasmid pYSF-AT1a by inserting the AT1a open reading frame between the EcoRI and SalI sites of pYSF1. To express rat B1 bradykinin receptor (B1R), we created plasmid pYSF-B1 by inserting the B1R open reading frame between the BglII and BamHI sites of pYSF1. Preparation of competent yeast cells and transformation was performed using a Fast-Yeast Transformation Kit (Geno Tech, Maryland Heights, MO, USA) according to the manufacturer's manual. Yeasts expressing the two GPCRs were prepared by a co-transformation of the plasmids.

Yeast reporter assay for mammalian GPCR and ligand binding

The yeast cells (1×10^5) were suspended with ligands in 40 µl of SD-ura medium (pH 7.0) in white 96-well flat-bottom microplates. After incubation, (5 h at 30°C), 40 µl of chemiluminescent substrate solution for the reporter β-galactosidase and a Gal-Screen System for yeast cell cultures (Applied Biosystems) was added to the reaction mixture and incubated an additional 1 h. Chemiluminescence was determined using an Applied Biosystems TR717 Microplate Luminometer.

Other materials

Gangliosides GT1b, GD1b, GD1a, GD3 and GM1 were purchased from Iatron Co. (Japan). The oligosaccharide portion of the ganglioside was prepared by EGCase digestion as described (*14*). Bradykinin, human ATII, B2R-selective antagonist Hoe140 and B1-selective antagonist Des-Arg^9-[Leu^8]-bradykinin were purchased from the Peptide Institute Co. (Japan).

Results

Bradykinin antagonist-associated inhibition of GT1b-mediated intracellular Ca^{2+} release, CaMKII activation and filopodia formation

We have previously shown that the oligosaccharide portion of GT1b ganglioside stimulates cdc42-mediated reorganization of cytoskeletal actin through the rapid release of Ca^{2+} from intracellular stores and through the activation of CaMKII (*1*). These findings suggest that oligosaccharides trigger cdc42 upregulation via a cell-surface receptor-like component, most likely GPCR. Although several downstream signals of cdc42 have been identified, only a few extracellular stimulators are known to activate cdc42 via GPCRs, one of which is bradykinin (*3–5*).

To determine whether bradykinin receptors act as oligosaccharide receptors, we investigated the effects of bradykinin antagonists on GT1b-induced CaMKII activation. We detected GT1b-induced CaMKII activation using a fluorescence microscopic imaging system and a fluorescent-labeled peptide substrate, AS2, as described previously (*1*, *9*). As shown in Fig. 1A, when GT1b was added to the extracellular media of primary cultured rat hippocampal neurons, a rapid increase in the fluorescence signal was detected in the cytosol, indicating that the substrate was

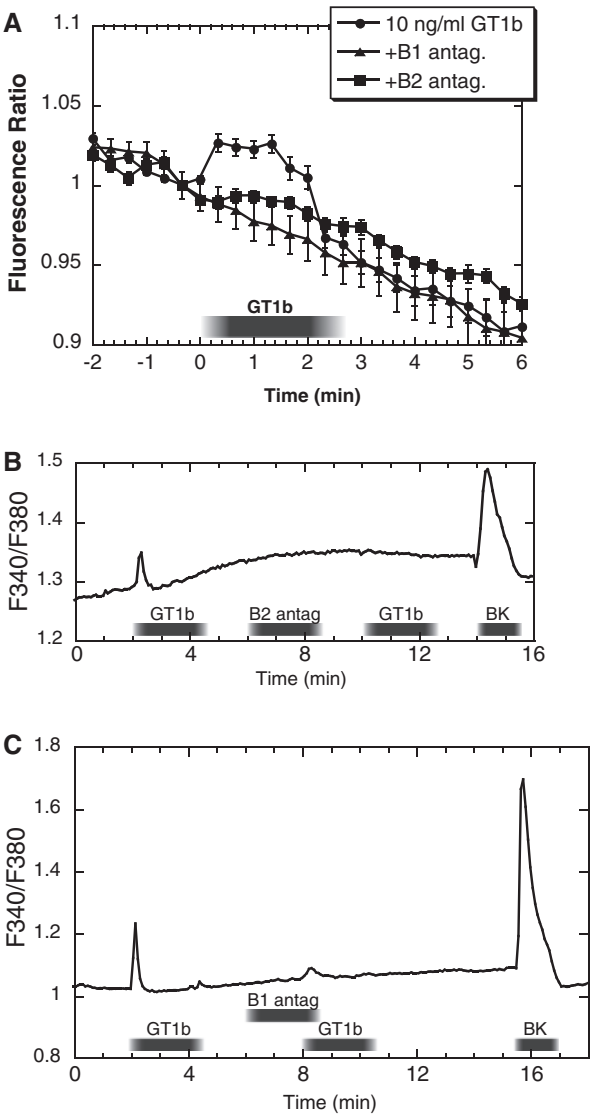

Fig. 1 Prevention of GT1b-mediated activation of intracellular CaMKII and Ca^{2+} release by bradykinin B1 and B2 antagonists. (**A**) Primary cultured rat hippocampal cells were preloaded with a fluorescent peptide substrate, AS2, and were exposed to 10 ng/ml GT1b for 2 min and 40 s as indicated. AS2 fluorescence increases as it becomes phosphorylated. The time course of the averaged fluorescence ratios in cytosolic areas of 28, 24 and 26 cells without an antagonist, with a B1 antagonist, and with a B2 antagonist, respectively, were plotted every 20 s. The ratio was calculated by dividing the fluorescence intensity at each time by that at −20 s. CaMKII activation by 10 ng/ml GT1b was undetectable in the presence of 100 nM B1 antagonist (Des-Arg^9-[Leu^8]-bradykinin) or 10 nM B2 antagonist (Hoe140) in the extracellular media. Error bars, standard error. (**B** and **C**) NG108-15 cells were preloaded with Fura-2-AM and were exposed to 20 ng/ml GT1b as in (A). The time course of the averaged fluorescence ratios excited at wavelengths of 340/380 nm (F340/F380) in cytosolic areas of 15 cells for (B) and 11 cells for (C) were plotted every 5 s. GT1b-induced elevation of intracellular Ca^{2+} levels were undetectable after exposure to 10 nM Hoe140 (B) and were inhibited after exposure to 100 nM B1 antagonist (C).

phosphorylated by CaMKII in accordance with a previous report (*1*). This reaction was inhibited when either a bradykinin B1 antagonist (100 nM Des-Arg^9-[Leu^8]-bradykinin) or a B2 antagonist (10 nM Hoe140) was present in the media. This result indicated that

GT1b-induced CaMKII activation was mediated by bradykinin B1 and/or B2 receptors. Since the upstream reaction of CaMKII is Ca^{2+} release from intracellular stores, we analyzed the effects of the antagonists on GT1b-induced elevation of intracellular Ca^{2+} levels. As shown in Fig. 1B and C, GT1b-induced Ca^{2+} release was inhibited after treating NG108-15 cells with B2 or B1 antagonists.

We then investigated how the antagonists affected filopodia formation, a downstream reaction of CaMKII activation. As shown in Fig. 2B and C, exposing NG108-15 cells to GT1b for 2 min induced filopodia formation in a CaMKII-dependent manner. The GT1b-mediated filopodia formation was partially blocked by the B1 antagonist and completely blocked by the B2 antagonist (Fig. 2D and E), indicating that the GT1b signal for filopodia formation was mainly mediated by B2Rs.

Neurite outgrowth on ganglioside-containing culture substrates and effects of B2 antagonist

We previously observed that the oligosaccharide portion of GT1b, when introduced to culture media, promoted dendritic outgrowth in primary cultured hippocampal and cerebellar Purkinje neurons by at least 4 days *in vitro* (DIV) (*1*). In the present study, we sought to determine whether these gangliosides coated on culture dishes affect early neurite development by contact with the cell surface. We prepared the ganglioside-containing culture substrate according to the method used for preparing chondroitin sulfate-containing culture substrates (*7*). The efficiency of ganglioside-coating was determined to be approximately 30%, meaning that about 300 ng of gangliosides was immobilized onto the glass surface (27 mm diameter) of a culture dish ($\approx$50 ng/cm^2). The ganglioside-containing culture substrate promoted neuritogenesis in primary cultured rat hippocampal neurons (Fig. 3). The longest neurite of cells was denoted axon-like neurites, whereas the remaining neurites were denoted dendrite-like neurites, according to Dotti *et al.* (*15*). GT1b was the most potent promoter of axon- and dendrite-like neuritogenesis followed by GD1b and GM1 (Fig. 4). When added to the culture medium, the B2 antagonist Hoe140 maximally inhibited GD1b- and GT1b-induced neuritogenesis at concentrations of 10 and 100 nM, respectively. The B1 antagonist also inhibited the neuritogenic effects of GT1b. These results indicate that gangliosides can potentially promote the growth of both axon- and dendrite-like neurites and that ganglioside signals are transduced via B2Rs and perhaps via B1Rs.

Gangliosides and their oligosaccharides stimulate B2Rs in a yeast reporter assay

To confirm the stimulatory effects of glycans on B2Rs, we used a yeast reporter system in which a yeast pheromone receptor, Ste2, was replaced with mammalian GPCR. In addition, the mammalian GPCR signal was linked via a chimeric yeast/mammalian Gα protein to the yeast pheromone receptor signaling pathway constructed to express the reporter β-galactosidase. The yeast/mammalian Gαq chimeric protein, GPA1-Gαq-c5, was constructed by replacing five amino acid residues in the Gpa1p C-terminal with those of Gαq. We examined glycan binding by either expressing B2R (GqB2) or coexpressing B2R with AT1a receptor (GqAB) in the yeast system. As shown in Fig. 5A, the oligosaccharide portion of GT1b reacted to both the B2R-expressing clones and the B2R/AT1a receptor coexpressing clones to activate the pheromone-signaling pathway. Yeast coexpressing B2R and AT1a reacted better than yeast expressing

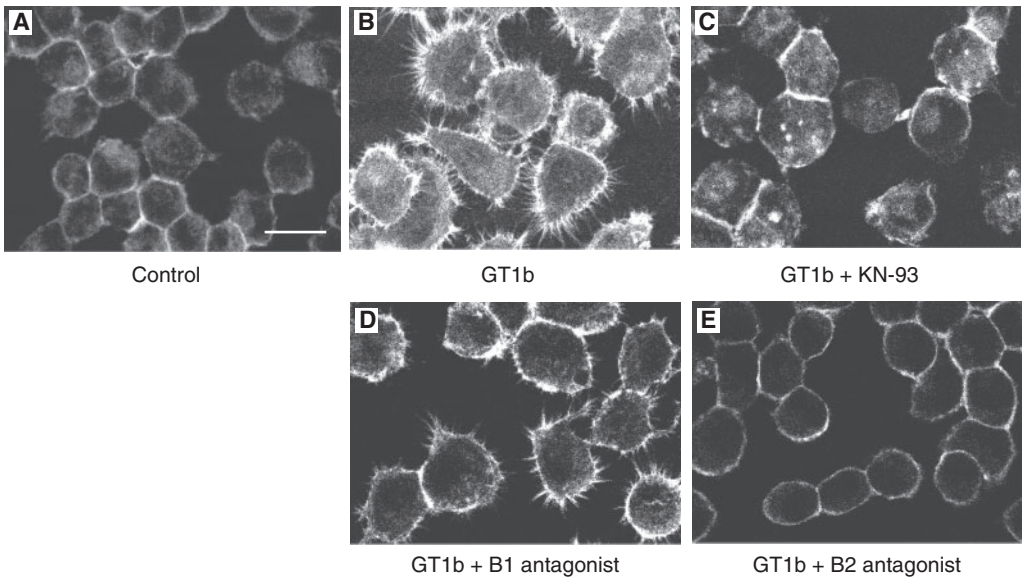

Fig. 2 Effects of CaMKII and bradykinin receptor inhibitors on GT1b-induced filopodia formation. NG108-15 cells were exposed to 10 ng/ml GT1b (**B–E**) without any inhibitors (**B**); or with 10 μM KN-93, a CaMKII inhibitor (**C**); 100 nM Des-Arg^9-[Leu^8]-bradykinin, a B1 antagonist (**D**); or 10 nM Hoe140, a B2 antagonist (**E**) for 2 min at 37°C. The cells were then fixed and stained with rhodamine–phalloidin and observed with a confocal fluorescence microscope. For the control, cells were untreated (A). Scale bar, 10 μm.

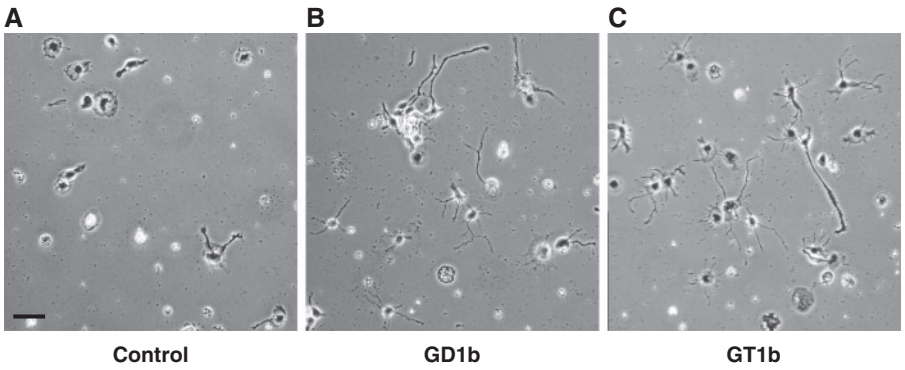

Fig. 3 Differentiation of primary cultured hippocampal neurons on glycan-containing substrate in the defined media. Axon- and dendrite-like neurites of neurons cultured on substrates containing GD1b (**B**) and GT1b (**C**) were more elongated than those cultured on the control poly-ornithine substrate (**A**). Defined culture media were used as described in 'Materials and Methods'. Scale bar, 50 μm.

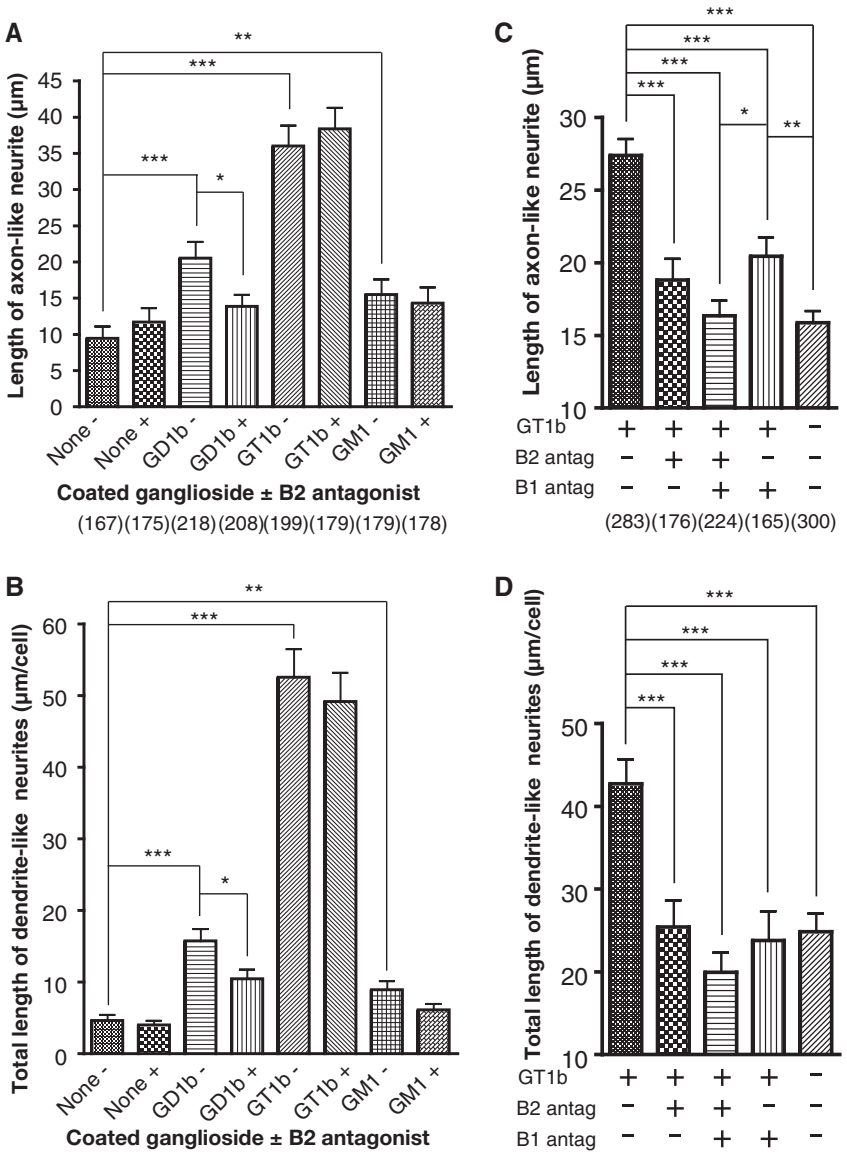

Fig. 4 Neurite outgrowth on glycan-containing culture substrates and effects of B2 antagonist. Hippocampal neurons were grown on substrates composed of the indicated gangliosides in culture media (see Fig. 3). (**A** and **B**) Effects of a B2 antagonist were examined by adding (+) 10 nM Hoe140 to the media. (**C** and **D**) The effects of a higher concentration (100 nM) of Hoe140 and 100 nM Des-Arg^9-[Leu^8]-bradykinin (a B1 antagonist) on GT1b-mediated neuritogenesis were examined in a separate experiment. A '+' or '–' denotes the presence or absence of GT1b coating or antagonist in the media, as indicated. The mean lengths ± SE of the longest neurites (termed 'axon-like neurites') (A and C) or the sum of lengths of other neurites (termed 'dendrite-like neurites') of single neurons (B and D) are plotted. Numbers of neurons analyzed are shown in parentheses in (**A**) and (**C**). Statistical significance of neurite length relative to different substrates and effects of antagonists were compared using a Student's t test. $^{***}P<0.001$; $^{**}P<0.01$; $^{*}P<0.05$.

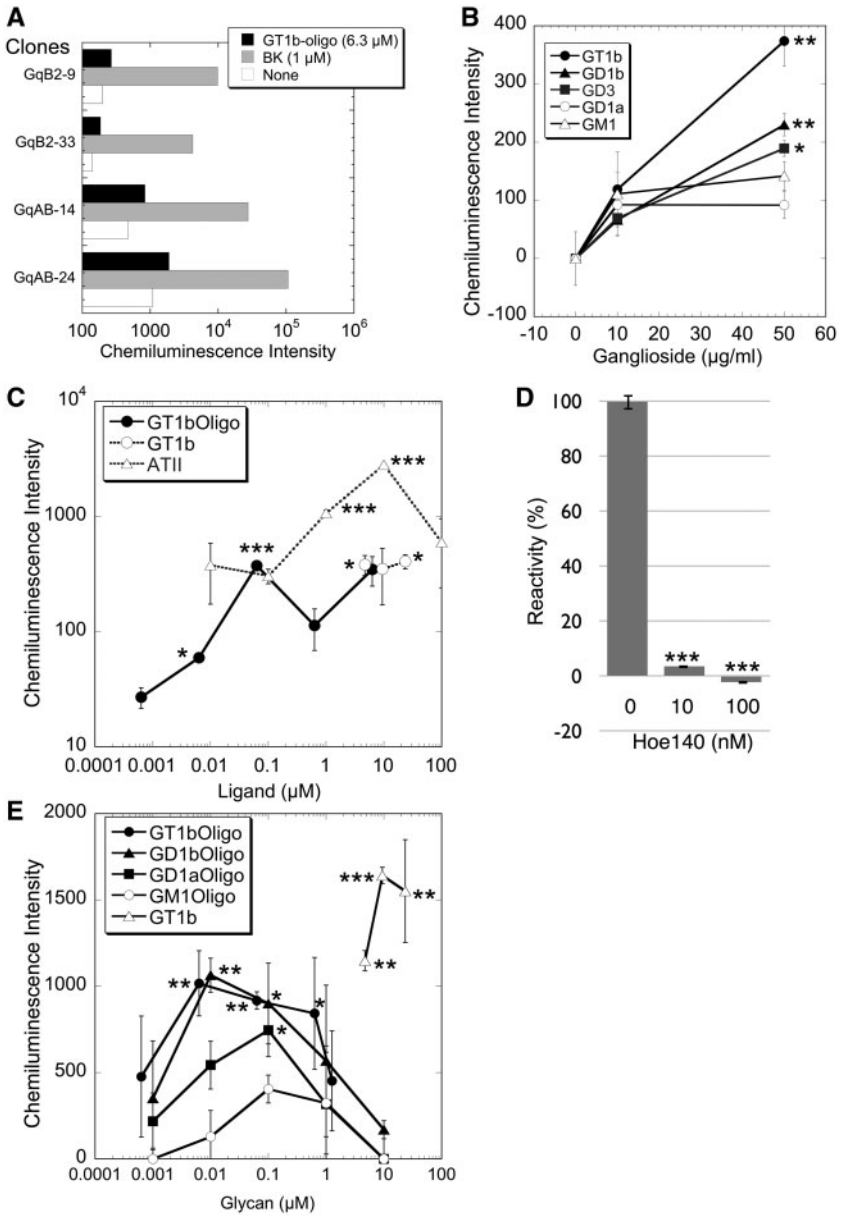

Fig. 5 Activation of B2R signal by exogenously applied glycans in yeast reporter assay. (**A**) Different yeast clones that expressed only human B2R (GqB2) or coexpressed B2R with human AT1a (GqAB) were used. Clone numbers are shown after the yeast names. Reactivity of the oligosaccharide portion of GT1b (GT1b oligo) and reactivity of 1 μM bradykinin (BK) are shown. (**B**) Reactivity of gangliosides GT1b, GD1b, GD3, GD1a and GM1 were determined using the yeast clone GqB2-9. Intensities of chemiluminescence produced by the β-galactosidase reporter represent reactivity. Error bars, standard error of three samples. (**C**) Reactivity of different concentrations of GT1b, GT1b oligosaccharide and human ATII to yeast clone GqAB-14 (A) were determined. Chemiluminescence intensities of assay mixture without ligand were subtracted from those with ligand. (**D**) Reactivity of 63 nM of the oligosaccharide portion of GT1b to yeast clone GqAB-14 was determined in the absence or presence of the B2 antagonist Hoe140 at the indicated concentrations. Relative activities (%) based on samples lacking the antagonist are shown. Similar results were obtained when another yeast clone (GqAB-24) was used. (**E**) Reactivity of GT1b and oligosaccharide portions of gangliosides in yeast expressing mouse B2R. Error bars, standard error of three samples. Significant differences of reactivity compared with reactivity at zero ligand concentration are shown as $^{***}P<0.001$; $^{**}P<0.01$; $^{*}P<0.05$ (Student's *t*-test).

solely B2R. The oligosaccharide did not react to yeast clones that solely expressed either B1R or AT1a (data not shown). Moreover, the oligosaccharide portion of GM1 ganglioside did not react to B2R/AT1a- or B2R-expressing clones (data not shown). These results indicate that B2Rs mediated the glycan signal of GT1b.

Using a yeast reporter assay, we then examined the reactivity of various gangliosides. As shown in Fig. 5B, GT1b, GD1b and GD3 reacted to B2R, with GT1b exhibiting the most potent reactivity. GD1b and GD3 showed moderate and slight reactivity, respectively, corresponding to their efficiency for stimulating CaMKII and cdc42-mediated actin reorganization (*1*). Similar results were obtained with yeast clone GqAB-14 (data not shown).

Since in the yeast reporter assay system the effective ganglioside concentration was above the micromolar range (Fig. 5B), which is 2–3 orders of magnitude higher than that in experiments using mammalian

cells, we assumed that, by interacting with the yeast cell wall, the hydrophobic ceramide portion of the gangliosides should influence how the gangliosides affect the receptor in the yeast cell membrane. Thus, we examined the reactivity of GT1b and its oligosaccharide portion to B2R-expressing yeast. As shown in Fig. 5C, in GqAB yeast the oligosaccharide portion of GT1b reacted at a concentration range of 6.3–63 nM, reaching nearly maximal reactivity at 63 nM. To determine whether bradykinin antagonists inhibit this reaction in the yeast assay system, we examined the reactivity of the oligosaccharide portion of GT1b to GqAB yeast in the presence of the B2 antagonist Hoe140. Hoe140 completely abolished oligosaccharide reactivity (Fig. 5D), confirming that B2R is a potential mediator of glycan signaling related to the oligosaccharide portion of GT1b ganglioside.

Next, we analyzed the reactivity of other ganglioside-derived oligosaccharides using yeasts that only express B2R. As shown in Fig. 5E, the oligosaccharide portion of GT1b reacted significantly to these yeasts at a concentration range of 6.3–630 nM in the same way as it reacted to GqAB yeast, with a maximal reactivity at 6.3 nM. In the same fashion as GT1b oligosaccharide, the oligosaccharide portion of GD1b reacted to B2R significantly at a concentration range of 10–100 nM, and the oligosaccharide portion of GD1a reacted significantly at a concentration of 0.1 μM. However, although the oligosaccharide portion of GM1 reacted to these yeast cells, the reactions were not statistically significant at a concentration range of 1 nM to 10 μM. Nonetheless, these results show that the oligosaccharides reacted to B2Rs on yeast cells as well as on mammalian cells at a concentration of less than 1 μM.

Discussion

The present study demonstrated that B2Rs modulate neuritogenesis by serving as mediators for the glycan signal of GT1b and GD1b gangliosides. GT1b and GD1b have been shown to stimulate B2Rs to activate the Ca^{2+}/CaMKII signal transduction pathway (*1*). This is supported by the finding that a B2 antagonist inhibited several GT1b-induced reactions in neuronal cells, such as Ca^{2+} release, CaMKII activation and cdc42-mediated actin reorganization (*1*). We then demonstrated that at least part of the modulatory effects of these glycans on neuritogenesis resulted from the stimulation of B2Rs by GT1b and GD1b gangliosides. To analyze the functional interaction between B2Rs and the glycans of these two gangliosides, we performed a yeast reporter assay, which showed that GT1b, GD1b and GD3 stimulate B2Rs. B2Rs are ubiquitously and selectively expressed in neurons (*16*) and probably contribute to neuronal differentiation as glycan-signal mediators in addition to their role as a peptide receptor.

The budding yeast *S.cerevisiae* has only two species of GPCRs, and their signaling pathways are well characterized. For the present study, we utilized an assay system in which yeast pheromone GPCRs were replaced with mammalian B2Rs. This assay system was much simpler to use than those involving mammalian cells. Mammalian cells are not suitable for analyzing the functions of a single GPCR because mammals have more than 300 species of GPCRs, some of which function by forming heterodimers (*17–25*), making it difficult to clarify which specific GPCRs are expressed in a cell. Moreover, it is difficult to clarify the output of the signals because sometimes more than two Gα proteins work as partners of a single GPCR (*26*). In the yeast reporter assay used here, we were able to observe bradykinin activity clearly (Fig. 5A). Moreover, yeast coexpressing B2R and AT1a, but not yeast expressing solely AT1a, reacted with ATII (Fig. 5C), which was expected since B2R/AT1a dimer formation is essential for ATII signal transduction via AT1a (*22*).

In this assay, we demonstrated preference of b-series gangliosides over a-series gangliosides in the stimulation of B2R. This is consistent with the preference observed in neuronal cells. In the yeast reporter assay, the effective concentration of a ganglioside was much higher than that of its oligosaccharide portion (Fig. 5), as the cell wall of yeast may interfere with hydrophobic ligand access to B2Rs. GT1b and GD1b stimulated B2Rs at the same concentration ranges (Fig. 5B). GD3 also showed weak, but significant reactivity to B2Rs consistent with our previous findings that GD3 also activates CaMKII and promotes CaMKII-dependent filopodia formation (*1*). Oligosaccharide portions of GD1a and GM1 showed reactivity at concentrations of 0.1 μM in the yeast reporter assay (Fig. 5E), whereas GM1 but not GD1a ganglioside stimulates slight elevation of cytosolic Ca^{2+} of NG108-15 cells (*1*). This discrepancy may be caused by the different accessibility of the gangliosides and oligosaccharides to the plasma membranes and different physicochemical nature of plasma membranes between yeast and mammalian cells. In our yeast reporter assay, specificity of glycan reactivity was the same for yeast expressing solely B2R and those coexpressing B2R and AT1a (Fig. 5A). However, yeast expressing either B1R or AT1a as the sole GPCR did not react with the glycans. Moreover, the B1 agonist Des-Arg^{10}-Kallidin failed to stimulate reporter production in B1R-expressing yeast (data not shown), suggesting that another factor, such as heterodimer formation, is necessary for the functional expression of B1R. The reaction of the glycans with B2R was completely inhibited by the B2 antagonist Hoe140. Thus, we concluded that glycans stimulate B2R. B2R stimulation by the glycans tended to be more intensive when B2R was coexpressed with AT1a. This may be due to heterodimer formation by these receptors (*22*).

In the present study, to confirm that the gangliosides target cell surface molecules in order to induce activities such as neuronal differentiation, we investigated how the gangliosides affect hippocampal neurons cultured on a ganglioside-coated surface. This method mimics cell–cell or cell–extracellular environment interactions. In this experiment, GD1b and GT1b stimulated the formation of both axon-like and dendrite-like processes. Hoe140 significantly inhibited

these effects (Fig. 4). In this analysis, GD1b-induced effects were inhibited significantly by 10 nM Hoe140, but GT1b-induced effects were not and 100 nM Hoe140 was needed for the inhibition. It is probable that the efficiency of GT1b is too high to compete with 10 nM Hoe140 since the actual densities of the gangliosides coated on the culture surface (≈ 50 ng/cm^2) were relatively higher than that added as a solution (10 ng/ml). We previously found that the oligosaccharide portion of GT1b stimulates dendritogenesis in primary cultured rat hippocampal neurons 4–7 DIV after exposure to the oligosaccharide (*1*). The present results are consistent with this phenomenon, which we surmise to be also mediated by B2Rs. The GT1b effect was also significantly inhibited by 100 nM B1 antagonist (Fig. 4C, D), suggesting that another signaling cascade via B1R was involved. This premise was corroborated by the findings that the B1 antagonist inhibited GT1b-induced Ca^{2+} release, CaMKII activation and filopodia formation in other experiments using neuronal cells (Fig. 1 and 2D).

Mice lacking all ganglio-series gangliosides, which are characteristically found in the brain, are born as double null mutants of GM3 and GM2 synthases (*2*). They die soon after birth due to impaired CNS maturation and exhibit axon degradation and altered axon–glial interactions. This indicates that gangliosides mediate neuronal maturation and maintenance of neuronal architecture and function. In the literature, there is a plethora of functional studies that have investigated gangliosides at the cell or tissue level (*10*). We previously identified an additional cdc42-mediated neuritogenesis pathway triggered by GM2 ganglioside (*27*). In a cell-free system, gangliosides interact directly with calmodulin and calmodulin-dependent enzymes, including CaMKII, to regulate enzymatic activity (*28*, *29*). Gangliosides and oligosaccharides applied extracellularly, as in the present study, have difficulty in rapidly accessing cytosolic proteins like calmodulin and calmodulin-dependent enzymes. Since gangliosides bind to unique amphiphilic peptide structures of these proteins (*30*), it is likely that they may also bind such structures in B2R protein.

Gangliosides account for a large part of brain glycoconjugates and are characteristically found in the brain. Different species of gangliosides are distributed differentially in the brain. GD1b and GT1b, which activate cdc42 via CaMKII activation, distribute in the area of synapses (*31*, *32*). B2Rs are ubiquitously and selectively expressed in brain neurons. In the cerebellum, Purkinje cells are enriched with B2Rs more than other neurons such as granular neurons (*16*). GD1b and GT1b are not expressed in Purkinje cells but are well expressed in granular neurons (*31*). Purkinje cells and granular neurons expand their dendrites and their axons, respectively, to form synapses in the molecular layer. Thus, B2Rs and ganglioside ligands may play roles in synapse formation between Purkinje cell dendrites and granular neuron axons. Consistent with this notion is the finding that adding the oligosaccharide portion of GT1b into the extracellular medium stimulated dendritogenesis in primary cultured Purkinje and hippocampal neurons (*1*). In the present study, we demonstrated that adding either GT1b or GD1b to the culture substrate stimulated early neuritogenesis in primary cultured hippocampal neurons. The effects of the gangliosides were mediated by B2Rs. The same signal may play a role in dendritogenesis in Purkinje cells.

Generally, GT1b is expressed during a late developmental stage in the brain. However, in rat cerebellar cortex, GT1b expression starts at birth first in the molecular and internal granular layers and then with development in other layers, except the Purkinje cell layer (*33*). Moreover, GT1b expression in the molecular and internal granular layers continues throughout adulthood (*33*). GD1b expression occurs in all layers of the cerebellar cortex, except the Purkinje cell layer, starting at birth and continuing throughout adulthood. Thus, these gangliosides may affect neurite outgrowth during brain development.

B2R-deficient mice are born, mature normally and are fertile (*34*). They do, however, exhibit some disorders such as Na^+-dependent hypertension (*35*), short life span, abnormal mitochondrial physiology, abnormal morphology of Leydig cells in testes and reduced bone density (*36*). At present, with the exception of altered nociceptive responses (*37*), no neurological abnormalities have been reported in these mice. However, since B2Rs are ubiquitously and selectively expressed in neurons (*16*), the effects of B2R deficiency on neural systems should be analyzed carefully. As the B2R-mediated glyco-signal system mediates at least some of the differentiation and maturation processes in the CNS, gangliosides, B2 antagonists and B2 agonists, therefore may be useful in regenerative therapy.

Recent reports have shown that B2Rs sense fluid shear stress (*38*), suggesting that dynamic changes in membrane structure due to environmental influences affect the spatial conformation of GPCRs and their activation. Oligosaccharide portions of GT1b and GD1b modulate the conformation of B2Rs in membranes for activation either by binding to the receptors themselves or by interacting with an environmental component of B2R-containing membranes, the micro-domain 'raft'.

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Conflict of interest

None declared.

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