Regulated expression system for GD3 synthase cDNA and induction of differentiation in Neuro2a cells

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It was reported recently by our group that the transfection of GD3 synthase cDNA into Neuro2a cells, a neuroblastoma cell line, caused cell differentiation with neurite sprouting (Kojima et al., 1994; J. Biol. Chem., 269, 30451-30456). To further explore this phenomenon in detail, we applied tetracycline-regulated system to control the expression of GD3 synthase cDNA in Neuro2a cells. Under this system, the process of Neuro2a cell differentiation was rather slow, about 3 weeks of cell culturing in the absence of tetracycline was required for most cells to extend the neurite-like structures. The RNase protection assay indicated that the mRNA of GD3 synthase gene was first detected between 4 and 8 h after the gene was activated and kept at approximately the same level through the process. Furthermore, time-course analysis of total ganglioside expressions has shown that GD3 and GT1b gangliosides appeared on the cell surface early in the process and reached the maximum level around day 6. We also found that the amounts of GD3 and GT1b on the cell surface started to decrease after day 6 and returned gradually to the basal values after 3 weeks. On the other hand, GQ1b and GD1b were started to be synthesized at early stage and the amounts were continuously to increase through the whole Neuro2a morphological change process. In addition, time-course analysis by flow cytometry method for GD3 and GQ1b suggested that the conversions of simple gangliosides to more complex gangliosides may be required to induce the Neuro2a differentiation. Our results indicated that the combination of cDNA transfection and regulated gene expression is a powerful tool to study the function of glycolipids and should have a general application to the glyobiology field.

Key words: Neuro2a/GD3 synthase gene/tetracycline-regulated system/ganglioside/neurite-like structures

Introduction

Gangliosides are sialic acid-containing glycosphingolipids that synthesized by glycosyltransferases (Caputto et al., 1971; Paulson and Colley, 1989; van Echten and Sandhoff, 1993). Various gangliosides in great amount have been found in brain tissues suggesting their important functional roles among neuronal cells, especially in cellular and control of cell proliferation (Suzuki, 1965; Hakomori and Igarashi, 1995; Hirschberg et al., 1996). For example, GQ1b ganglioside was reported to promote neurite outgrowth in two types of human neuroblastoma cells, GOTO and NB-1 (Tsuji et al., 1983; Nakajima et al., 1986). Also, differentiation of mouse neuroblastoma Neuro2a cells and rat pheochromocytoma PC12 cells induced by exogenous addition of various gangliosides in the absence of NGF has been reported previously (Tsuji et al., 1988a,b; Ledeen et al., 1990; Mutoh et al., 1995).

GD3 synthase (α2,8-sialyltransferase) gene has been cloned by several groups including ours from human melanoma cDNA library (Haraguchi et al., 1994; Nara et al., 1994; Sasaki et al., 1994). This enzyme plays a key role in the biosynthesis of b- and c-series gangliosides. Recently, we have shown that by transfection the GD3 synthase cDNA into Neuro2a cells induces expression of not only GD3 but also b-series gangliosides (Scheme 1). More interestingly, the transected Neuro2a cells show differentiation with neurite sprouting (Kojima et al., 1994). To gain a better understanding of the consequences of GD3 synthase expressing in Neuro2a cells and explain the possible mechanism responsible for the Neuro2a morphological change, we applied the recently developed tetracycline regulated system (Gossen et al., 1993) to GD3 synthase cDNA. Tetracycline-regulated system is an inducible gene expression system that place target gene under the control of a regulatory sequence from the tetracycline-resistance operon of Tn10 (Gossen and Bujard, 1992; Gossen et al., 1993; see Figure 1, top). When a chimeric tetracycline-controlled transactivator (TtA), a hybrid fusion protein combines the tet-repressor DNA-binding domain with the transcriptional activation domain of herpes simplex virus protein 16 (VP16), binds to the minimal promoter, the target gene (GD3 synthase cDNA) should be activated. In the presence of tetracycline, the binding of the tetracycline with the tTA prevents GD3 synthase cDNA activation by causing a conformational changes in the tet-repressor. In this study, we analyzed the time-course of GD3 mRNA and total ganglioside expressions, especially GD3 and GQ1b, for stable transfected Neuro2a cells. Our strategy proved to be a useful way to study the functions of glycolipids in cellular differentiation and growth.

Results

GD3 synthase cDNA expression in tTA-Neuro2a cells was regulated by tetracycline

In order to control the GD3 synthase cDNA expression in Neuro2a cells by the tetracycline system, plasmid pCMViTA-Hygro (Figure 1, bottom) was stable transfected into the Neuro2a cells and colonies were selected by the presence of hygromycin (150 μg/ml) in the medium. Twelve hygromycin-resistant colonies were isolated and expended into the DMEM medium. All of them were stable transfected again using second plasmid pTetMCS-GD3-Neo (Figure 1, bottom). Cells were cultured in the presence of hygromycin (150 μg/ml) and neomycin (800 μg/ml), colonies were chosen randomly, and
Our ribonuclease protection assay results indicated that the mRNA for GD3 synthase cDNA was detected between 4 h and 8 h after the gene was activated, see Figure 2. At 4 h, the transcript was barely detected. This delayed mRNA expression pattern could be due to the characteristics of tetracycline system (Gossen et al., 1992). Interestingly, the mRNA was maintained at about the same level through the whole tTA-Neuro2a differentiation process, that was from normal Neuro2a morphology to the one with extended neurite-outgrowth (see below). Compared to the GAPDH mRNA level, GD3 synthase mRNA was synthesized and maintained at lower level, as shown in Figure 2.

**Differentiation of tTA-Neuro2a cells induced by the expression of GD3 synthase cDNA**

Under the control of tetracycline system, tTA-Neuro2a cells changed their morphology and extended axon-like structures. But this process was a rather slow one, see Exp. 1 in Figure 3. The neurite outgrowth of some tTA-Neuro2a cells was first observed around day 5 (panel B) after the GD3 synthase cDNA was activated by the removal of tetracycline in the culture medium. The term "neurite" used here was defined as a cell with a pseudopod which exceeds one or more cell diameters in length. This morphological change process was continued gradually and the neurite outgrowth became more and more extended, as shown in panels C and D. At day 20, the majority of tTA-Neuro2a cells became neuron-like cells, panel E. On the other hand, two control experiments including double stable transfected Neuro2a cells without GD3 synthase cDNA (panels F and G) and with GD3 synthase cDNA but cultured in the presence of tetracycline in the medium (panel H) have shown that the morphology of these cells was basically the same as the parental Neuro2a cells. These results indicated that GD3 synthase cDNA expression was solely responsible for the induction of neurite outgrowth of tTA-Neuro2a cells.

To gain a better understanding of the neurite structures, the neurites were immunostained with the SM131 monoclonal antibody. SM131 binds to the phosphorylated neurofilaments present in axon but not in dendrites (Sternberger and Sternberger, 1983; Lichtenberg-Kraag et al., 1992). As shown in Figure 3, Exp. 2 (panels J and K), the neurites were immunostained strongly with SM131 at day 20, indicating they were axon-like structures. The cell nuclei were also strongly stained reflecting the nonspecific binding using this antibody (panel I).

To distinguish the final tTA-Neuro2a cell morphology was due to real differentiation or some kind of adaptation, we stained the cells for acetylcholine esterase (Lebel et al., 1994) and the result was shown in Figure 4. When tetracycline was presented in the culture medium, very small amount of acetylcholine esterase expression was detected. On the other hand, at the stage of day 20 without tetracycline in the culture medium, strong expression of acetylcholine esterase was detected. To get an idea on cell proliferation rate for tTA-Neuro2a cells at several stages, a cell proliferation assay was performed using CellTiter assay, see Materials and methods. This assay is based on the cellular conversion of a tetrazolium salt into a formazan product that is detected using an ELISA plate reader. Figure 5 demonstrated that the cell proliferation rate was decreased almost 50% at day 20 comparing to 0 h.

We also tested the possibility that the differentiated tTA-Neuro2a cells could reverse their morphology when tetracycline was added again to the culture medium and therefore GD3 synthase cDNA expression was suppressed. The result
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Fig. 1. Tetracycline inducible gene expression system. (Top, a) In the presence of tetracycline, it bound to a chimeric transactivator (tTA) that was constitutively expressed in tTA-Neuro2a cells and prevented tTA binding to the tetO sequences, so the promoter was silent. (b) In the absence of tetracycline, tTA bound to tetO sequences and GD3 synthase cDNA was activated. (Bottom) Two plasmids in the tetracycline regulated system. Plasmid pCMVtTA-Hygro expressed the tTA regulator protein from the strong immediate early promoter of cytomegalovirus. A hygromycin resistance gene was contained in this plasmid. Plasmid pTetMCS-GD3-Neo expressed the GD3 synthase cDNA from the minimal CMV promoter. A neomycin resistance gene was contained in this plasmid.

indicated that the morphology of differentiated tTA-Neuro2a cells was irreversible.

Time-course study for total gangliosides of tTA-Neuro2a

During the process of tTA-Neuro2a differentiation induced by the expression of GD3 synthase cDNA under the control of tetracycline system, gangliosides from eight stages of cells were purified and analyzed by thin-layer chromatogram, Figure 6. At day 0, when GD3 synthase cDNA expression was suppressed by tetracycline presented in the medium, the ganglioside expression pattern from tTA-Neuro2a cells was almost the same as control Neuro2a cells, both cell lines expressed significant amounts of a-series gangliosides. When GD3 synthase cDNA was activated by the removal of tetracycline from the medium, a dramatic ganglioside expression pattern change was observed, mainly the conversion of a-series to b-series gangliosides. GD3 synthase cDNA was activated by the removal of tetracycline from the medium, a dramatic ganglioside expression pattern change was observed, mainly the conversion of a-series to b-series gangliosides. At day 1, small amounts of GD3, GD1b, GT1b, and GQ1b were detected by TLC and GD1a amount was markedly decreased. This a-series to b-series ganglioside conversion was continued through day 10, except GT1b amount started to decrease after day 3. After day 10, GD3 amount started to decrease but GQ1b amount continued to increase. It was interesting to notice that there seemed to be a ganglioside content equilibrium of tTA-Neuro2a cells from day 3 to day 10.

To further examine some ganglioside changes in more detail, we measured four ganglioside (GD3, GD1b, GT1b, and GQ1b) content changes by using the densitometry based on the TLC data, see Figure 7. The results suggested that the amounts of GQ1b and GD1b changed in a similar way after GD3 synthase cDNA was activated. Both ganglioside amounts increased steadily through the process. After about 3 weeks, the combined percentage of GQ1b and GD1b amounts in total gangliosides was >65%. On the other hand, GD3 and GT1b ganglioside also changed in a similar pattern after the gene was activated. Both ganglioside amounts reached the peak points around day 6 and then decreased gradually to the basal Neuro2a levels after about 3 weeks culturing in the absence of tetracycline in the medium. The combined percentage of GD3 and GT1b amounts in total gangliosides was less than 10%.

Time-course analysis of GD3 and GQ1b gangliosides by flow cytometry

Since GD3 and GQ1b expression patterns changed significantly when GD3 synthase cDNA was activated and there seemed to be some correlation between tTA-Neuro2a differentiation and GD3, GQ1b amount changes, we measured both gangliosides by staining the cells with monoclonal antibodies against GD3 and GQ1b, respectively, and followed by flow...
Fig. 2. Ribonuclease protection analysis of GD3 synthase mRNA expression during the course of tTA-Neuro2a differentiation. Ten micrograms of total RNA was hybridized to biotin labeled RNA probe (1 ng/ml) of GD3 synthase cDNA and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) cDNA. Following digestion of single-stranded RNA, the protected probe was resolved on an acrylamide gel.

cytometry analysis. tTA-Neuro2a cells were cultured and collected at several time points after GD3 synthase cDNA was made active. These time points included: 0 h, 3 h, 16 h, 24 h, day 3, day 5, day 7, day 10, day 16, day 23, and day 28. Hour 0 was defined as the point when the culture medium containing tetracycline (2 µg/ml), and the GD3 synthase cDNA expression was suppressed. For the purpose of clarity, flow cytometry analysis data from six representative time points (0 h, 16 h, day 3, day 10, day 16, and day 25) were shown in Figure 8. In the case of GD3 (Figure 8a), at 0 h, the amount on the cell surface was about the same as control Neuro2a cells in negligible amounts. But GD3 synthesis increased gradually and reached a high level at day 3 after tetracycline was removed from the culture medium and was kept approximately at that level until day 10. Amount of GD3 started to decrease at a slow but steady rate after 10 day culturing without tetracycline. GD3 ganglioside continued to decrease to the control Neuro2a level all the way to about day 23 when the majority of the tTA-Neuro2a cells started to extend the axon-like structures. On the other hand, for GQ1b ganglioside (Figure 8b), there was a small increase of GQ1b synthesis on cell surface compared to the control Neuro2a cells at 0 h. That could be due to the leakage caused by small percentage of cells that were controlled more loosely by the tetracycline system. When the suppression for GD3 synthase cDNA was lifted by removal of tetracycline from the medium, the GQ1b peak on flow cytometry analysis started to shift from the control Neuro2a peak gradually, and this trend was kept continuously through the whole process. A second peak for GQ1b of the flow cytometry data could be seen starting from about the 3 day point, indicating the uneven amounts of GQ1b presented among cell population. The flow cytometry results correlated well with the TLC data.

Discussion

In this study, we used the neuroblastoma cell line Neuro2a as a model system to study the functions of gangliosides on cell morphological change and neurite outgrowth. We examined the changes and roles of cell surface gangliosides by transfection and expression of the GD3 synthase cDNA, which encodes a key enzyme for ganglioside synthesis. The expression of GD3 synthase cDNA was controlled by the recently developed tetracycline system. Under this system, the Neuro2a cells changed to the neuron-like cells with the characteristic phenotype of differentiated neurons with neurite extensions.

The tetracycline system is a newly developed inducible gene expression system that overcomes many of the limitations encountered by other systems, including basal leakiness and toxic effects by inducer (Gossen et al., 1993). Although the system works well, there are still problems of low levels of expression for the inducible gene and sometimes uneven expression among controlled cells (Gossen et al., 1992). In our tetracycline system for GD3 synthase cDNA in Neuro2a cells, this low expression level was confirmed by the RNase protection assay (see Figure 2).

There were several indications suggesting that the final morphological change for tTA-Neuro2a was a stage of cell differentiation. First of all, at morphological level, tTA-Neuro2a at late culturing stage extended neurite-like structures, and it was immunostained positive using SMI31 antibody, which binds to the phosphorylated neurofilaments present in axon but not in dendrites. Secondly, tTA-Neuro2a cells cultured without tetracycline in the medium at late stages showed decreased cell proliferation and expressed acetylcholine esterase, indicating differentiation of the cells. Furthermore, the neurite outgrowth
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Fig. 3. Morphology and immunofluorescence microscopy of tTA-Neuro2a cells. Exp. 1, cells were double stable transfected with pCMVtTA-Hygro and pTetMCS-GD3-Neo or pCMVtTA-Hygro and pTetMCS-Neo, as described under Materials and methods. (A–E) show the morphology of tTA-Neuro2a cells transfected with pCMVtTA-Hygro and pTetMCS-GD3-Neo at 0 h, day 5, day 10, day 15, and day 20. (F) and (G) show the morphology of tTA-Neuro2a cells transfected with pCMVtTA-Hygro and pTetMCS-Neo at 0 h and day 20. (H) shows the morphology of tTA-Neuro2a cells transfected with pCMVtTA-Hygro and pTetMCS-GD3-Neo at day 20, but cells were grown in the medium supplemented with 2 μg/ml tetracycline. Exp. 2, tTA-Neuro2a cells were stained with antibody against phosphorylated epitope in neurofilament, SMI31. Panel I shows the immunofluorescence microscope of tTA-Neuro2a cells at 0 h. (J) and (K) show the immunofluorescence microscope of tTA-Neuro2a cells at day 20 for two regions.

It has been known for some time that exogenous addition of gangliosides causes the morphological changes of cultured cells and the process is rapid, within a day (Tsuji et al., 1983, 1988a,b). Under the tetracycline system, GD3 synthase cDNA transfected tTA-Neuro2a cells changed their morphology in a much slower manner; it took about 3 weeks for the majority of cells to extend their neurites after the GD3 synthase cDNA was activated. Although the underlying mechanism was unclear to us at this stage, there were two possible explanations for this phenomenon, one was that (1) the GD3 synthase mRNA was expressed and kept at a low level during the process under the tetracycline system. The slow turnover of gangliosides might be due to the low GD3 synthase mRNA expression level. As indicated by Figures 6 and 8, changes in the expression levels and types of gangliosides marked the beginning for tTA-Neuro2a cell morphological change to neuron-like cells with

Fig. 4. Acetylcholine esterase staining for tTA-Neuro2a cells when tetracycline was present in the culture medium (0 h) and when tetracycline was absent in the culture medium (day 20).
characteristic phenotypes. The conversion of simple gangliosides, e.g., GD3 to more complex ganglioside GQ1b may be essential for the process of Neuro2a cell differentiation. (2) It was possible that some unidentified signal transduction system, which links to some transcription activation system related to neurite formation, was responsible for tTA-Neuro2a morphological change under the tetracycline system. Recently, we have identified two new genes that were involved in this process by differential display method. Both genes seemed to be developmentally regulated in the mouse brain (Liu and Tsuji, unpublished observations).

It was shown recently that GD3 and GT3 are synthesized by a single enzyme in human and therefore GD3 synthase is also GT3 synthase (Nakayama et al., 1996). However, we found recently that only driving by strong promoters, such as CMV promoter, GD3 synthase gene behaved as the GT3 synthase gene (unpublished observations). In this study, we also did flow cytometry analysis on tTA-Neuro2a cells using monoclonal antibody against GT3. Our results (data not shown) indicated that there was not any GT3 synthesized during the process of tTA-Neuro2a morphological change. Therefore, in our system, GD3 synthase enzyme did not show the capacity as GT3 synthase. Also, the synthesis of GD3 and other b-series gangliosides was a slow process under our system. We also notice some difference when comparing the present tetracycline regulated system to our previous nonregulated system (Kojima et al., 1994). In the previous system, because the gene expression was not controlled and there was no timing process involved, GD3 expression was also detected in addition to the GQ1b expression. This could also be due to the different promoters used to drive the GD3 synthase cDNA.

There is some evidence suggesting Trk A protein (Mutoh et al., 1995), ceramide (Riboni et al., 1995), and consequent MAPK activation may be responsible for the differentiation of Neuro2a cells. Although the underlying molecular mechanism remains poorly understood, there is suggestion that GM1 mimics the effects of NGF and activates the Trk A signal transduction. We also measured the NGF levels for tTA-Neuro2a cell medium at several culturing stages, including both 0 h and day 20 cell stages. The result indicated that NGF did not change significantly at different stages, suggesting other mechanism for this process (H. Liu, S. Furukawa, and S. Tsuji, unpublished observations).

Several studies have shown that the expression of gangliosides during brain development are strictly regulated (Yu et al., 1992).
1988; Svennerholm et al., 1991; Rosner et al., 1992), GD3 is expressed at the early stage of development, during neural tube formation and neuroblast and glioblast proliferation. At the late stage of development, that is at neurogenesis and neuritogenesis stage, diminished expression of GD3 and increased synthesis of other b-series gangliosides including GQ1b are observed. Our time-course study based on the flow cytometry analysis for GD3 and GQ1b and TLC analysis on several gangliosides during the process of Neuro2a differentiation also indicated the similar ganglioside change pattern.

Taken together, our results indicated that stable transfection of glycocoytransferase cDNA into neuroblastoma cell lines modifies the de novo biosynthesis of gangliosides; in conjunction with the gene regulated system, it is a powerful tool to study the functions of various gangliosides on cell fate at defined stages of cell development and the signal transduction pathway involved.

Materials and methods

Plasmid constructions

The original tetracycline-inducible expression plasmids pUHD 10–3 (pTetMCS) and pUHD 15–1 (pCMVtTA) were generously provided by H. Bujard (Gossen et al., 1992). The pTetMCS plasmid contains a cytomegalovirus (CMV) minimal promoter linked to seven repeat units of the enhancer and the tetR-VP16 fusion. To construct the plasmid pTetMCS-GD3-Neo, complete GD3 cDNA was amplified by PCR with primers 5’ CCTAGACTCTTGTGCTGAGGCC 3’ and 5’ TTCTTGACTTTATT- GTTCTCAGAAGATTG 3’ (underlines indicated synthetic BglII restriction sites) using pUC119-GD3 (Sasaki et al., 1994) as template. The PCR fragment was digested with BglII and inserted into the BamHI site of pTetMCS. A 1.13 kb fragment containing TK promoter, neuromycin gene, and polya signal was excised from pMCIneoPOLA (Stratagene) and was ligated into the Xhol site of pTetMCS-GD3. The final plasmid obtained was named pTetMCS-GD3-Neo. To construct the plasmid pCMVtTA-Hygro, a 1.7 kb fragment containing the TK promoter, complete Hygromycine gene, and polya signal was amplified by PCR with primers 5’ GTCTGGCCCAAGCTTGCAGTGGAT 3’ and 5’ AACCCCCGGAGCTTATATACACAGC 3’ (underlines indicated synthetic HindIII restriction sites) using p3’SS (Stratagene) as template. The PCR fragment was digested with HindIII and inserted into the HindIII site of pCMVtTA. The final plasmid obtained was named pCMVtTA-Hygro.

Transfection, generation of stable transformed cells

Murine neuroblastoma Neuro2a cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO-BRL, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) and antibiotics at 37°C in 5% CO2. Plasmid pCMVtTA-Hygro was transfected into a 60-mm-diameter plate of Neuro2a cells using the LipofectAMINE reagent (GIBCO-BRL) following the manufacturer’s recommended protocol. Colonies were selected in the presence of Hygromycin (150 μg/mL). Twelve colonies were picked randomly and expanded, transfected again with the plasmid pCMVtTA-Hygro reagent. Colonies were selected again in the presence of G418 (800 μg/mL, GIBCO) as well as Hygromycin (150 μg/mL) but without tetracycline in the culture medium. Colonies were screened by flow cytometry analysis using monoclonal antibody against GD3. Twenty-four Hygromycin and G418-resistent colonies were picked up randomly and expanded into culture medium. Eight of them showed strong GD3 expression on the cell surface. All eight colonies were then cultured in the medium containing tetracycline (2 μg/mL) for 10 days and analyzed by flow cytometry again staining the cells with GD3 antibody. Three of them showed strongly controlled GD3 expression pattern by tetracycline. One of them was chosen randomly and used for the study of this article. This cell line was named tTA-Neuro2a.

Flow cytometry analysis, immunofluorescence microscopy, and cell proliferation assay

The detailed procedures for flow cytometry analysis and immunofluorescence microscopy were described previously (Kojima et al., 1994). Briefly, for flow cytometry analysis, cells were harvested with 0.02% EDTA solution and washed with PBS containing 1% bovine serum albumin and 0.05% NaN3 and then treated with GD3 antibody. Three of them showed strongly controlled GD3 expression pattern by tetracycline. One of them was chosen randomly and used for the study of this article. This cell line was named tTA-Neuro2a.
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cyteometry. with a FTTC-labeled goal F(ab')2 fragment of anti-mouse IgG and anti-mouse IgM mixture (1:40 dilution) for 30 min at 4°C and then subjected to flow

cells were treated with 20 ng/ml mouse IgG1. After washing with PBS containing 1% bovine serum albumin and 0.05% NaN3, cells were treated

tTA-Neuro2a cells at several stages were

tTA-Neuro2a cell; NGF, nerve growth factor; PBS, phosphate-buffered saline; GPDH, glyceraldehyde-3-phosphate dehydrogenase; mAb, monoclonal antibody; N2a,

Acetycholine esterase staining of cells

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Abbreviations

DMEM, Dulbecco's modified Eagle medium; FACS, fluorescence-activated cell sorting; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mAb, monoclonal antibody; N2a, Neuro2a cell; NGF, nerve growth factor; PBS, phosphate-buffered saline; PCR, polymerase chain reactions; TLC, thin-layer chromatogram; tTA-Neuro2a, double-stable transfected tetracycline regulated Neuro2a cell line. The nomenclature used for gangliosides and glycosyltransferases is based on the abbreviations according to Svennerholm (1965).

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