

N-acetylglucosaminyltransferase VB expression enhances β 1 integrin- dependent PC12 neurite outgrowth on laminin and collagen

Intaek Lee,¹ Hua-Bei Guo, Maria Kamar, Karen Abbott, Karolyn Troupe, Jin-Kyu Lee, Gerardo Alvarez-Manilla and Michael Pierce

The Complex Carbohydrate Research Center and Department of Biochemistry and Molecular Biology, the University of Georgia, Athens, Georgia, USA

Abstract

N-acetylglucosaminyltransferase VB (GnT-VB, -IX) is a newly discovered glycosyltransferase expressed exclusively in high levels in neuronal tissue during early development. Its homolog, GnT-V, is expressed in many tissues and modulates cell–cell and cell–matrix adhesion. The ability of GnT-VB to regulate cell–matrix interactions was initially investigated using the rat pheochromocytoma PC12 neurite outgrowth model. PC12 cells stably transfected with GnT-VB consistently showed an enhanced rate of nerve growth factor (NGF)-induced neurite outgrowth on collagen and laminin substrates. Levels of TrkA receptor phosphorylation and downstream ERK activation induced by NGF were not influenced by GnT-VB expression. No significant difference was observed in the

rate of neurite outgrowth when cells were cultured on non-coated culture dishes, indicating that integrin–ECM interaction is required for the stimulatory effects. Neurite outgrowth induced by manganese-dependent activation of β 1 integrin on collagen and laminin substrates, however, showed a significant increase in neurite length for the PC12/GnT-VB cells, compared with control cells, suggesting that the enhancement is most likely mediated by alteration of β 1 integrin–ECM interaction by GnT-VB. These results demonstrate that GnT-VB expression can modulate the rate of neurite outgrowth by affecting β 1 integrin–ECM interaction.

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The cloning of a neuronal-specific homolog of UDP-N-acetylglucosamine : α (1,6)-D-mannoside β (1,6)-N-acetylglucosaminyltransferase (GnT-V, Mgat5), designated N-acetylglucosaminyltransferase VB (GnT-VB or GnT-IX) has recently been reported (Inamori *et al.* 2003; Kaneko *et al.* 2003). GnT-VB was shown to be highly expressed in brain and testis selectively, while GnT-V was expressed broadly in many tissue types. When transfected in a CHO variant cell line lacking functional GnT-V (Lec4 CHO), GnT-VB expression caused increased surface binding of L-phytohemagglutinin (L-PHA), suggesting that GnT-VB was capable of synthesizing N-linked glycoproteins expressing Gal β (1,4)-GlcNAc β (1,6)-Man α (1,6) similar to the original GnT-V (Cummings and Kornfeld 1982). An *in situ* staining study of GnT-VB mRNA in developing mouse embryos revealed that GnT-VB is selectively expressed in the brain and neural retina at day 8, suggesting that GnT-VB may function in neuronal differentiation and migration during brain development (Matthews *et al.* manuscript in preparation).

During N-linked glycan biosynthesis, GnT-V transfers UDP-N-acetylglucosamine (GlcNAc) to the α (1,6) mannose of tri-mannosyl core in β (1,6) linkage, and this branch is known in many cell types to be a preferred acceptor for the formation of poly N-acetylglucosamine chains (Shoreibah *et al.* 1993). Increased GnT-V expression has been shown in

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Address correspondence and reprint requests to Michael Pierce, Complex Carbohydrate Research Center, University of Georgia, Athens, GA 30602–4712, USA. E-mail: hawkeye@uga.edu

¹The present address of Intaek Lee is the Department of Internal Medicine, School of Medicine, Washington University, St Louis, MO 63110, USA

Abbreviations used: BSA, bovine serum albumin; ECM, extracellular matrix; FBS, fetal bovine serum; GlcNAc, N-acetylglucosamine; GnT-V, N-acetylglucosaminyltransferase V; L-PHA, L-phytohemagglutinin; Man, mannose; MAP1, microtubule-associated protein 1; NGF, nerve growth factor; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate – polyacrylamide gel electrophoresis.

several experimental systems to stimulate cell motility and to contribute to tumor cell invasion and metastasis in various malignant cancers (Dennis *et al.* 1987; Fernandes *et al.* 1991; Guo *et al.* 2002). When GnT-V activity is reduced or eliminated, cell adhesion is significantly enhanced (Guo *et al.* 2005). The effects on cell adhesion by GnT-V involve both integrin- and cadherin-based cell–matrix and cell–cell adhesion, respectively (Guo *et al.* 2002, 2003). As GnT-VB is selectively expressed in neuronal cells, we hypothesized that GnT-VB likely displays a similar function to affect cell–matrix interactions to enhance neuronal migration and axonal extension of developing neurons during neuromorphogenesis. As an initial test of this hypothesis, we utilized the rat pheochromocytoma subline, PC12, to determine the effects of increased GnT-VB expression on nerve growth factor (NGF)-induced neurite outgrowth in culture. The results showed that expression of GnT-VB in PC12 cells (PC12/GnT-VB) results in a significantly enhanced rate of neurite outgrowth after NGF treatment, compared with mock-transfected cells. Expression of GnT-VB did not cause a change in NGF-TrkA signaling nor in downstream ERK signaling. Addition of a cAMP activator, forskolin, known to induce neurite outgrowth in a PC12 variant line, PC12D, failed to initiate neurite outgrowth in either mock- or GnT-VB-transfected PC12 cells, suggesting that GnT-VB-transfected PC12 cells differ from PC12D cells in terms of mechanism of the ‘priming’ of neurite extension (Kato-Semba *et al.* 1987; Sano *et al.* 1990). The enhanced rate of neurite migration in NGF-treated PC12/GnT-VB cells was observed on either collagen- or laminin-coated culture plates, but there was no significant difference when cells were plated on non-coated culture plates. Manganese treatment has also been shown to stimulate neurite outgrowth through an integrin-dependent pathway. The enhanced rate of neurite outgrowth on collagen-coated plates seen in the PC12/GnT-VB cells could also be observed when cells were activated by treatment with manganese instead of NGF (Lin *et al.* 1993). Addition of $\beta 1$ integrin function-blocking antibody caused complete abolition of NGF- and manganese-induced neurite outgrowth in both control and PC12/GnT-VB cells. Furthermore, increased $\beta(1,6)$ branched *N*-linked glycans were detected on $\beta 1$ integrin in three different neuronal cell lines, including PC12 cell, following GnT-VB transfection. These results demonstrate that GnT-VB expression significantly accelerates NGF- and manganese-induced neurite extension on collagen and laminin, and this acceleration likely involves effects on $\beta 1$ integrin–matrix interactions.

Experimental procedures

Cell culture, transfection, and materials

Rat pheochromocytoma PC12, human glioma U373, and rat gliosarcoma 9L cells were purchased from ATCC (Manassas,

VA, USA) and grown in RPMI1640 medium supplemented with 10% horse serum, 5% fetal bovine serum on collagen I-coated 100-mm dishes (BD Biosciences, Bedford, MA, USA). Nerve growth factor-induced differentiation was typically performed on collagen I- or Laminin-coated 6-well plates or chamberslides under low (2%) serum conditions for the indicated times. Human GnT-VB/pcDNA3.1 expression plasmid was prepared as described previously (Kaneko *et al.* 2003). Lipofectamine 2000 (Life Technologies, Rockville, MD, USA) was used for stable transfection of human GnT-VB into PC12 cells following standard transfection procedures provided by the manufacturer. Cells were co-transfected with pEGFP vector (Invitrogen, Carlsbad, CA, USA) with 5 GnT-VB : 1 pEGFP ratio and selected for 3 weeks under 800 $\mu\text{g}/\text{mL}$ G418. Cells with the highest GFP expression were isolated using fluorescent activated cell sorting (Dakocytometer MoFlo). The 2.5S nerve growth factor (NGF) was obtained from Chemicon (Temecula, CA, USA). Biotinylated *L*-phytohemagglutinin (*L*-PHA) was from Vector Laboratories (Burlingame, CA, USA). Rabbit anti-phospho-TrkA (Tyr490) antibody was purchased from Cell Signaling Technology (Beverly, MA, USA). Rabbit anti-TrkA antibody, anti-ERK2 antibody, anti-phospho-ERK1/2 antibody, and all secondary antibodies conjugated with horseradish peroxidase were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti- $\beta 1$ integrin antibody, mouse monoclonal antibody against microtubule-associated protein 1 (MAP1), and anti-rat $\beta 1$ integrin function-blocking antibody, Ha2/5, were obtained from Chemicon. Streptavidin-conjugated phycoerythrin was from Sigma (St Louis, MO, USA), while forskolin was purchased from Calbiochem (San Diego, CA, USA).

Flow cytometry analysis

PC12 cells were harvested by trypsinization and incubated in culture medium at 37°C for 1 h to recover cell surface proteins. Cells were then fixed with 2% formaldehyde in phosphate-buffered saline (PBS) for 15 min, washed three times with PBS, and blocked with PBS containing 1% bovine serum albumin (BSA) and 0.01% sodium azide for 30 min at 4°C. Cells were then incubated with biotinylated *L*-PHA (1 $\mu\text{g}/\text{mL}$) in blocking buffer for 30 min at 4°C. After washing three times with blocking buffer, streptavidin-conjugated phycoerythrin (1 : 1000) was used to detect *L*-PHA bound to cell surface glycoproteins by incubating for 30 min at 4°C. After washing three times with blocking buffer, cells were then analyzed by FACSCaliber (Becton Dickinson Immunocytometry systems, San Jose, CA, USA).

Immunoprecipitation and western blot analysis

Cells were lysed in a buffer consisting of 1% Triton X-100, protease inhibitors and 0.1% sodium dodecyl sulfate (SDS). Typically, 100 μg total protein was used for immunoprecipitation and lectin precipitation. Proteins were resolved by SDS–polyacrylamide gel electrophoresis (PAGE) utilizing 7.5% or 4–20% gels under non-reducing conditions unless otherwise indicated, transferred to polyvinylidene difluoride membranes, blocked in 2% BSA, and probed with antibodies. Following incubation with horseradish peroxidase-conjugated secondary antibodies, proteins were detected by the enhanced chemiluminescence reagent (Pierce, Rockford, IL, USA). For detection of TrkA and ERK1/2 phosphorylation, the blot was probed with anti-TrkA Tyr-490 (1 : 1000) and anti-TrkA

antibody or anti-phospho-ERK1/2 (1 : 500) and anti-ERK2 antibody and developed by enhanced chemiluminescence. Densitometry of the blots was performed using a Bio-Rad Fluor S imager (Bio-Rad Laboratories, Hercules, CA, USA).

Treatment of PC12 cells with NGF or manganese

Typically, cells were plated on collagen-coated 6-well plates or chamberslides (BD Biosciences) overnight. After 24 h, cells were washed once with PBS, culture medium was replaced with medium containing 2% FBS supplemented with NGF (50 ng/mL) or manganese (200 μ M), either in the presence or absence of anti- β 1 integrin function-blocking antibody, Ha2/5 (10 μ g/mL). Normal rabbit IgG was used as a control. For longer periods of incubation, the NGF-treated media was replaced every 3 days.

GnT-V activity assay

Cells were trypsinized, pelleted, and lysed with 50 mM 2-(N-Morpholino)ethanesulfonic acid (MES) (pH 6.5), 150 mM NaCl, and 1% Triton X-100. Insoluble debris was pelleted by microcentrifugation (10 min at 4°C), and the supernatant was used for the GnT-V activity assay using GlcNAc β (1,2)Mana(1,2)Glc-O-octyl as an acceptor substrate and UDP-[3 H]-GlcNAc in the presence of Mn $^{2+}$. Sep-Pak columns (Waters, Milford, MA, USA) were used to separate the substrates and products as described (Shoreibah *et al.* 1992). The column methanol eluant was added to scintillation fluid and radioactivity measured by scintillation counting. Assays were performed in duplicate, the data averaged, and results were expressed as specific activity (nmol/h/mg). Protein concentration in the cell lysates was determined using the bicinchoninic acid (BCA) assay (Pierce).

Measurement of neurite outgrowth

Typically, cells were plated on collagen-coated plates and cultured overnight. NGF (50 ng/mL) was added to the cells in culture medium containing 2% serum. After stimulation for the indicated times with NGF, cells were washed and fixed with 3.5% formaldehyde. Ten random fields were photographed with a phase contrast microscope at 100 \times magnification. A bar that is as long as the average diameter of cell body in a field was placed in each picture and used as a standard to determine the length of neurites from each cell. Approximately a total of 300 cells were measured for neurite lengths, and cells with different lengths of neurites were grouped together such as one-cell body long, two-cell body long, and three-cell body long. Experiments were repeated twice for confirmation.

Results

Expression of GnT-VB caused an increased surface binding with L-PHA

PC12 cells stably expressing human GnT-VB were established as described in Experimental procedures and used to determine if GnT-VB expression resulted in increased β (1,6) branched *N*-linked glycans on the cell surface. Using an enzymatic assay with radiolabeled UDP-[3 H]GlcNAc, 10 mM Mn $^{2+}$ and the synthetic trisaccharide acceptor, GlcNAc β (1,2)Mana(1,2)Glc-O-octyl, PC12 transfected with

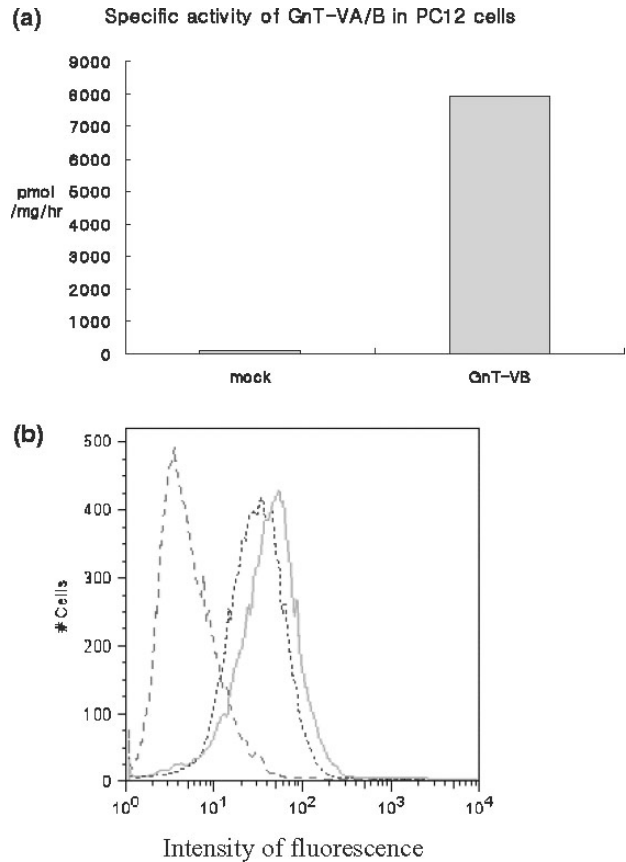


Fig. 1 PC12 cells stably transfected with GnT-VB show increased enzymatic activity and cell surface binding by L-PHA. (a) Enzymatic assay using radiolabeled UDP-[3 H]GlcNAc and synthetic trisaccharide acceptor in the presence of Mn $^{2+}$ (b) Flow cytometry analysis of PC12 cells expressing GnT-VB using L-PHA. Cells were incubated with biotinylated L-PHA, followed by streptavidin-phycoerythrin, as described in Experimental procedures (long-dashed line, mock-transfected PC12 cells stained with PBS only; short-dashed line, mock-transfected PC12 cells stained with L-PHA-phycoerythrin; bold line, GnT-VB-transfected PC12 cells stained with L-PHA-phycoerythrin).

GnT-VB (PC12/GnT-VB) showed a large increase in activity (Fig. 1a), compared with mock-transfected cells, while the parental PC12 cells showed very low endogenous GnT-V and GnT-VB activities. Flow cytometry analysis using fluorescent L-PHA indicated increased β (1,6) branched *N*-linked glycans on the surface of PC12/GnT-VB, compared with control cells (Fig. 1b), suggesting that GnT-VB is capable of producing *N*-linked glycans in PC12 cells that are bound by L-PHA, similar to the results seen with CHO Lec4 cells expressing GnT-VB (Kaneko *et al.* 2003). As GnT-VB is also capable of synthesizing β (1,6) branched structures on *O*-mannosyl-linked peptides (Inamori *et al.* 2004), it is likely that increases in the expression of these structures may also result from GnT-VB expression.

PC12 cells expressing GnT-VB showed stimulation of NGF-induced neurite outgrowth

To test whether GnT-VB expression in PC12 cells would affect NGF-induced neurite outgrowth, both mock-transfected and PC12/GnT-VB cells were plated on collagen-coated 6-well culture plates and treated with 50 ng/mL NGF for up to 14 days. The rate of neurite outgrowth was examined and measured at day 3, 7, and 14 using phase-contrast microscopy, as described in Experimental procedures. Interestingly, PC12/GnT-VB cells showed a significantly enhanced rate of neurite outgrowth, compared with control cells (Fig. 2a). Initial neurite outgrowth of PC12/GnT-VB was detected between 36 and 48 h, compared with approximately 72 h for control PC12 cells.

Moreover, the rate of neurite extension was significantly increased in the GnT-VB-expressing cells. When cells were counted in separate groups, such that cells with different neurite lengths (i.e. two cell-bodies, three cell-bodies, four cell-bodies) were grouped individually for analysis, PC12/GnT-VB showed much higher percentages of cells with neurites of two, three, or four cell-body length, compared with mock-transfected cells (Fig. 2b). Overall, quantitative analysis based on the ratio of lengths of neurites relative to cell-body length indicated that PC12/GnT-VB cells showed more than triple the percentage of cells with at least two-body length neurites at day 7 (Fig. 2c) compared with mock-transfected cells. After day 14, no significant differences in neurite lengths were observed, suggesting that

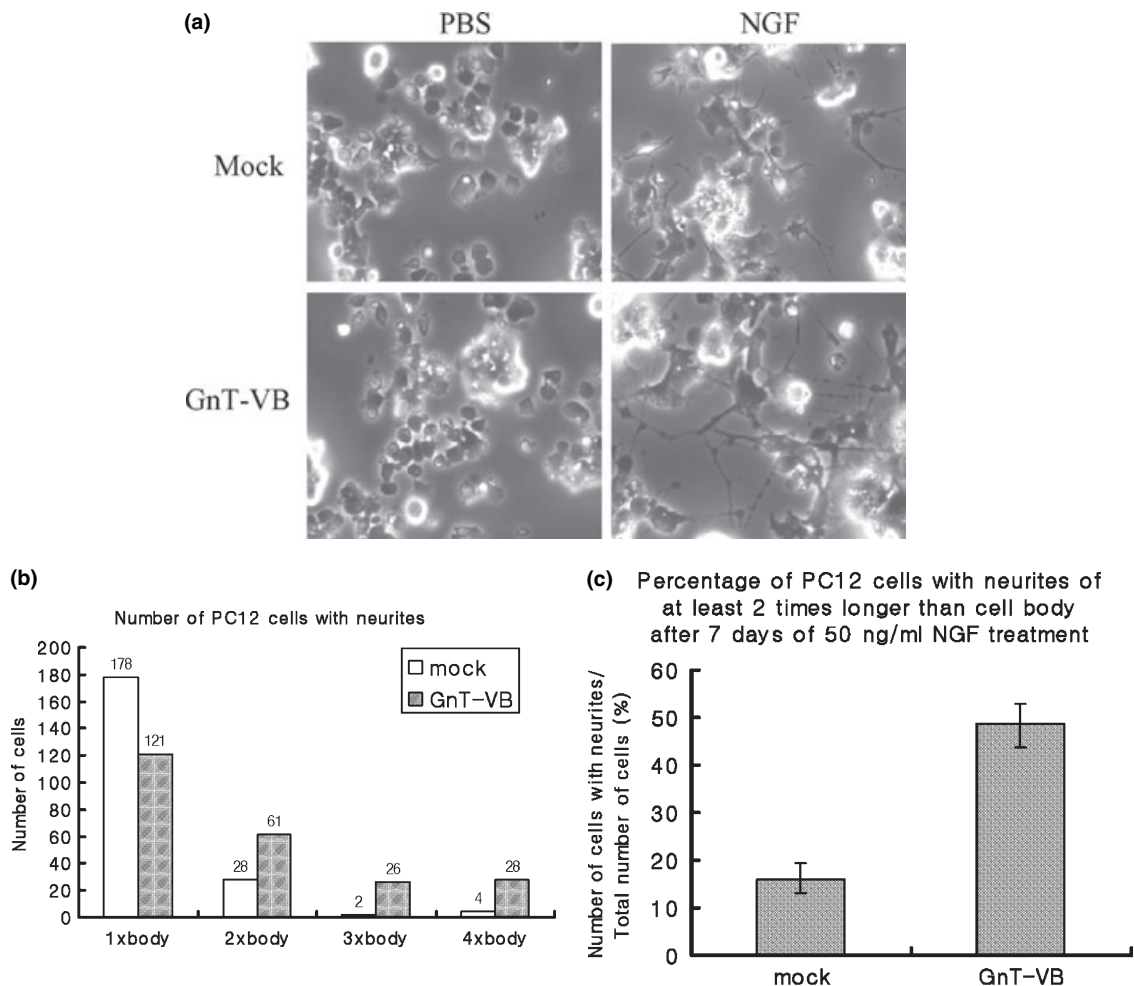


Fig. 2 GnT-VB expressing PC12 cells show enhanced NGF-induced rates of neurite outgrowth on collagen substrates, compared with mock transfected PC12 cells. (a) Cells were plated on 6-well plates coated with collagen I and cultured overnight, followed by treatment with either PBS or NGF (50 ng/mL) in culture medium containing 2% serum. The difference in neurite outgrowth was most prominent between day 5 and day 7, and both mock- and GnT-VB transfected

PC12 cells showed maximal neurite outgrowth after day 14. (b) Cells with different lengths of neurites were quantitatively analyzed, as described in Experimental procedures. Two independent experiments were carried out to confirm the result. The numbers shown in the figures were averaged from two experiments. (c) Percentage of cells with neurites that are at least twice the length of cell body. All data were obtained at day 7.

GnT-VB modulates the rate, but not the extent of neurite outgrowth. In addition, the number of neurites from each cell stayed about the same; on average, both mock- and GnT-VB-transfected PC12 cells developed approximately 4–5 neurites/cell when stimulated with NGF. These results indicated that increased GnT-VB expression led to stimulation of neurite extension in NGF-induced PC12 cells. We next investigated possible mechanisms for this stimulatory effect of GnT-VB expression on rate of neurite outgrowth.

Expression of GnT-VB did not influence NGF-TrkA and downstream ERK signaling

A neurotrophin receptor, TrkA, is highly expressed in PC12 cells and functions as an NGF receptor that, upon ligand binding, activates a downstream signaling pathway leading to ERK activation, an essential signaling intermediate for NGF-induced PC12 neurite outgrowth (Morooka and Nishida 1998; Xie *et al.* 2000; Nusser *et al.* 2002). We hypothesized that the enhanced neurite outgrowth observed in PC12/GnT-VB cells may be mediated by alteration of NGF-TrkA signaling. To test this hypothesis, cells were plated on collagen-coated dishes, treated with increasing doses of NGF, and levels of TrkA and ERK phosphorylation were measured. The results showed that addition of NGF yielded a similar dose-dependent response of TrkA phosphorylation of Tyr490 (Fig. 3; upper panel) in both mock- and GnT-VB-transfected cells. The level of ERK1/2 phosphorylation also did not show significant differences (Fig. 3; lower panel), suggesting that the NGF-induced signal transduction pathway was not likely a significant contributor to the enhanced neurite outgrowth seen in PC12/GnT-VB cells. These results showed that GnT-VB expression did not have a significant effect on NGF-TrkA and downstream ERK signaling in PC12 cells.

Nerve growth factor, but not forskolin, induced neurite outgrowth in both mock- and GnT-VB transfected PC12 cells

Enhanced neurite outgrowth in the absence of NGF treatment has been described for a PC12 variant, PC12D (Heumann *et al.* 1983; Sano *et al.* 1990), which has been characterized as being 'primed' for neurite extension. In addition, PC12D cells appeared much better spread on culture dishes compared with PC12 cells. Two unique features of this 'priming' effect on neurite outgrowth in PC12D cells have been characterized, including (i) increased expression of MAP1 compared with conventional PC12 cells, likely promoting faster microtubule assembly (Drubin *et al.* 1985); (ii) induction of neurite outgrowth by a cAMP activator, forskolin, in PC12D but not PC12 cells (Kato-Semba *et al.* 1987; Sano *et al.* 1990). To test if PC12/GnT-VB showed 'primed' behavior similar to PC12D cells, we investigated whether addition of forskolin can induce neurite extension. The results showed that treatment with forskolin (10 μ M) for 7 days failed to induce either any significant neurite outgrowth or MAP1 expression in either GnT-VB- or mock-transfected cells (data not shown). These results suggested that GnT-VB-dependent enhancement of NGF-mediated neurite outgrowth is likely distinct from that which is operative in the PC12D cells.

GnT-VB-mediated enhancement of neurite outgrowth required integrin–ECM interaction

There have been reports that neurite outgrowth of neuronal cells is directly mediated by integrin–ECM interactions in the developing nervous system, as well as in the PC12 neurogenesis model system (Ivins *et al.* 2000; Wildering *et al.* 2002; Guan *et al.* 2003). To test if PC12/GnT-VB cells showed enhanced neurite outgrowth in the absence of

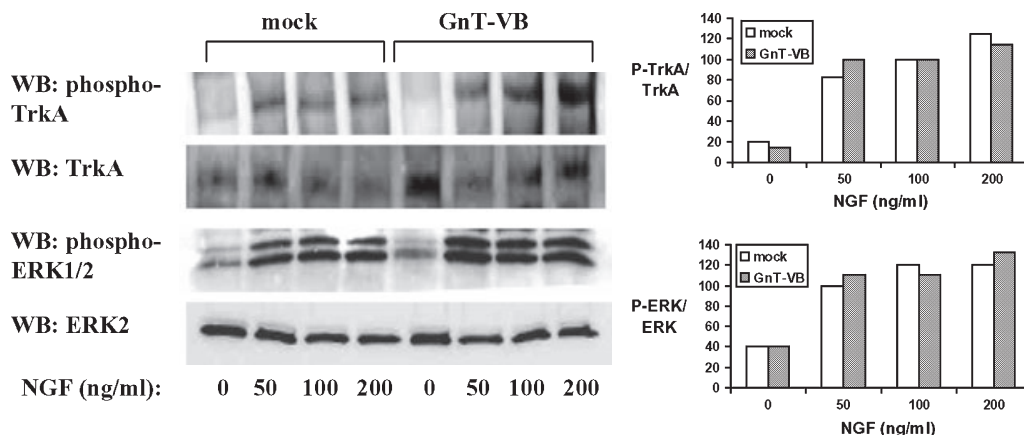


Fig. 3 NGF-TrkA and downstream ERK signaling are not affected by GnT-VB expression. Cells were plated on collagen-coated 100-mm dishes and cultured overnight. Culture medium was replaced with medium containing 2% serum and incubated for 18 h before indicated amounts of NGF in serum-free medium were added to the cells for

10 min. Cells were then harvested and subjected to SDS-PAGE and western blotting (WB) with either anti-TrkA and phospho-TrkA (Tyr490) antibody or anti-phospho-ERK1/2 and anti-ERK2 antibody, respectively. Densitometries of the blots were performed as described in Experimental procedures.

exogenous ECM, cells were plated on non-treated tissue culture plates or plates coated overnight with either collagen or laminin and then treated with NGF for 7 days. Surprisingly, on non-treated plates, PC12/GnT-VB cells showed no significant difference in the rates of neurite outgrowth, compared with control cells (Figs 4a and b), while PC12/GnT-VB cells plated on either collagen or laminin showed a significant enhancement of NGF-induced neurite outgrowth, suggesting that integrin–ECM interactions are critical for this stimulation. Furthermore, addition of the $\beta 1$ integrin function-blocking antibody, Ha2/5 (10 $\mu\text{g}/\text{mL}$) completely abolished NGF-induced neurite outgrowth of both control and GnT-VB expressing cells grown on both collagen- and laminin-coated wells (Figs 4a and b). No significant inhibition was observed with addition of control normal rabbit IgG

(data not shown). These results suggested that enhanced neurite outgrowth of PC12 cells by GnT-VB expression is most likely mediated by a $\beta 1$ integrin–ECM-dependent mechanism.

Enhancement of neurite outgrowth mediated by manganese-induced $\beta 1$ integrin activation of PC12/GnT-VB cells

The regulation of neurite outgrowth by integrin activation has been well documented (Lin *et al.* 1993; Ivins *et al.* 2000; Lein *et al.* 2000; Wildering *et al.* 2002). In particular, the manganese-dependent activation of $\beta 1$ integrin has been shown to promote neurite outgrowth in PC12 cells (Lin *et al.* 1993). Therefore, we studied if activation of $\beta 1$ integrin by manganese treatment was sufficient for PC12/

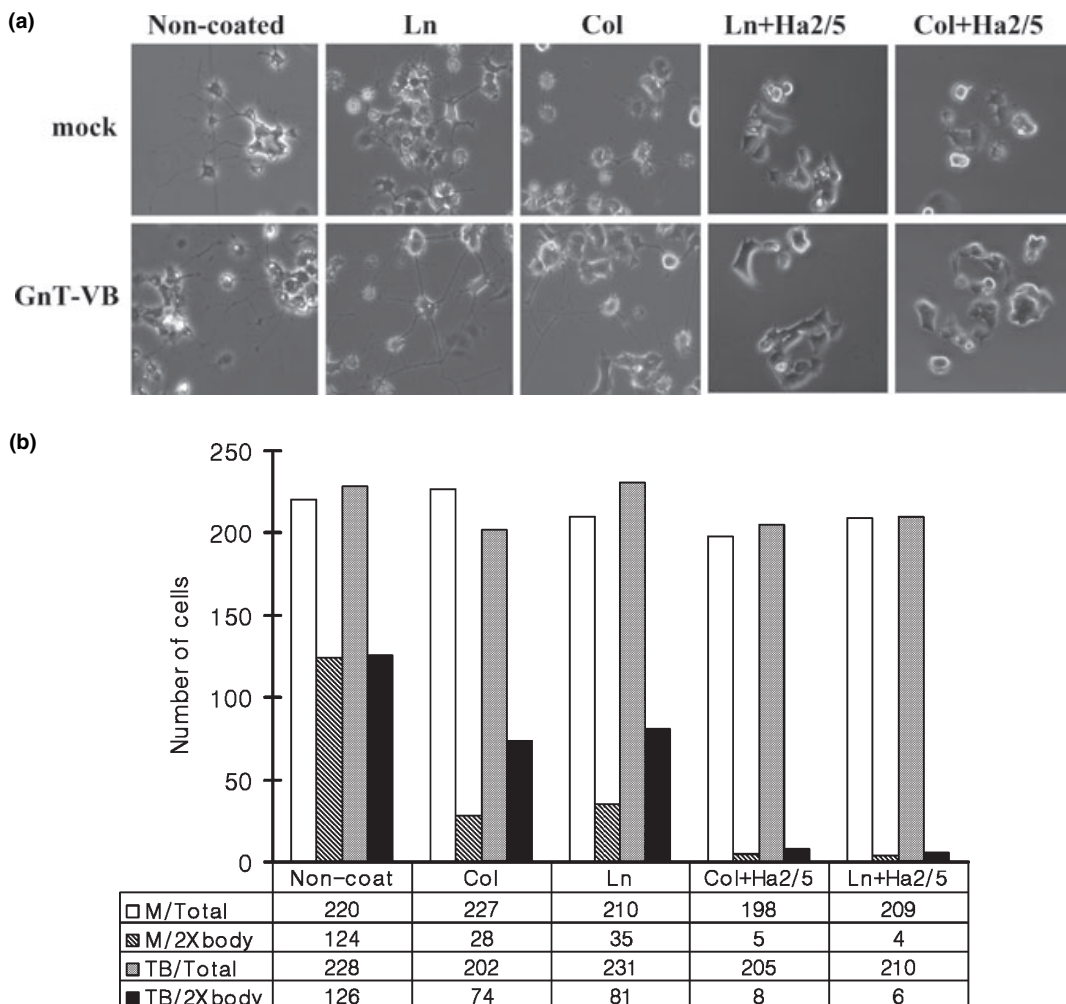


Fig. 4 Enhanced neurite outgrowth of PC12/GnT-VB requires $\beta 1$ integrin–collagen/laminin interaction. (a) PC12/GnT-VB cells show no significant difference in NGF-induced neurite outgrowth when cultured on non-treated tissue culture plates. Presence of Ha2/5 causes complete inhibition of NGF-induced neurite outgrowth in both collagen- and laminin-coated tissue culture plates. Cells were plated on culture

plates coated with collagen or laminin and cultured overnight. After 24 h, cells were stimulated with NGF either in the presence or absence of Ha2/5, as described in Experimental procedures. (b) Quantitative analysis of cells with neurites at least twice longer than cell body at day 7 of NGF treatment. Col, collagen; Ln, laminin; M, mock; TB, GnT-VB.

GnT-VB cells to show enhanced neurite outgrowth on collagen substrates, compared with control cells, as was seen by the treatment with NGF. Cells were plated on collagen-coated plates and treated with manganese for 72 h in the presence or absence of $\beta 1$ integrin function-blocking antibody. The results from this experiment showed that manganese-induced neurite outgrowth on a collagen substrate was significantly enhanced in the PC12/GnT-VB cells, and outgrowth was inhibited by addition of anti- $\beta 1$ integrin function-blocking antibody (Fig. 5a). In control PC12 cells, only small processes and occasional short neurites were observed in response to the manganese treatment, compared with the well-developed neurites of the PC12/GnT-VB cells. When the number of cells expressing neurites was quantified, PC12/GnT-VB cells showed more than twice the percentage of cells with one body-length neurites, compared with control cells (Fig. 5b), providing additional support for the hypothesis that GnT-VB expression alters $\beta 1$ integrin–collagen interaction which, in turn, leads to enhanced neurite outgrowth.

Increased $\beta(1,6)$ branched *N*-glycans on $\beta 1$ integrin in neuronal cells transfected with GnT-VB

We next examined whether $\beta 1$ integrin was a substrate for GnT-VB in PC12 cells and two other neuronal cell lines, human glioma U373 and rat gliosarcoma 9L cells, using lectin precipitation and western analysis using $\beta 1$ integrin antibody. Cell lysates from control and GnT-VB expressing cells were first precipitated with L-PHA to bind $\beta(1,6)$ branched *N*-linked glycans. Bound glycoproteins were then subjected to SDS-PAGE and blotted onto a polyvinylidene difluoride membrane. When probed with anti- $\beta 1$ integrin antibody, the data indicated that L-PHA bound significantly more $\beta 1$ integrin in all three cell types transfected with GnT-VB (PC12, U373, and 9L), demonstrating that $\beta 1$ integrin is a substrate for GnT-VB when it is expressed in neuronal cells (Fig. 6; right panel). After transfection of all three cell types, no significant differences in $\beta 1$ integrin protein expression levels were detected by western blot analysis (Fig. 6; left panel). Taken together, these results suggest that GnT-VB expression promotes enhanced neurite outgrowth by altering $\beta 1$ integrin–ECM interactions, rather than by affecting the NGF-TrkA and downstream signal transduction pathway.

Discussion

Our results showed that expression of GnT-VB in PC12 cells promotes enhanced NGF-induced neurite outgrowth on collagen and laminin substrates, and that this enhancement may be mediated by alteration of $\beta 1$ integrin function in its interaction with these ECM ligands. As GnT-VB is strongly expressed in the developing mouse embryo brain (Matthews *et al.* manuscript in preparation), it is of particular interest that GnT-VB is capable of stimulating neurite extension. It

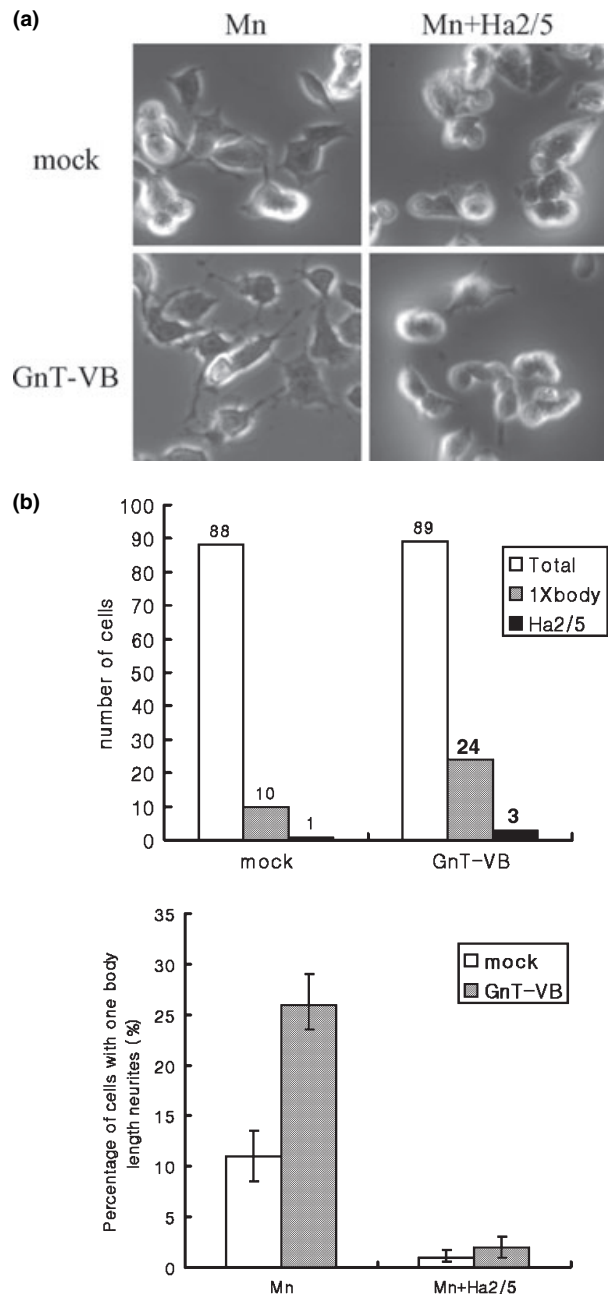


Fig. 5 Enhanced NGF-induced neurite outgrowth can be mimicked by neurite outgrowth induced by manganese-dependent activation of $\beta 1$ integrin in PC12/GnT-VB cells. (a) PC12/GnT-VB shows enhanced neurite outgrowth after manganese induction, compared with mock transfected PC12 cells. Cells were plated on collagen-coated plates and stimulated with 200 μM MnCl_2 for 72 h. (b) Quantitative analysis of manganese-induced neurite outgrowth. Six random fields from duplicate wells were photographed, and numbers of cells with one-body length neurites were quantitated.

has been shown recently that integrin–ECM interaction plays an important role in axonal extension during neuronal differentiation (Guan *et al.* 2003; Kiryushko *et al.* 2004). It

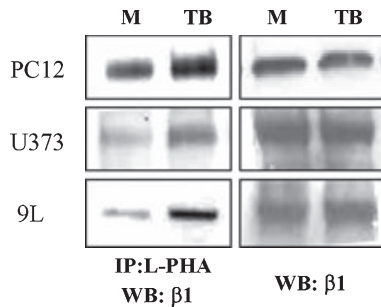


Fig. 6 $\beta 1$ integrin is a substrate of GnT-VB in neuronal cells. Expression of GnT-VB in PC12 cells, human glioma U373 and rat gliosarcoma 9L caused increased $\beta(1,6)$ branched *N*-linked oligosaccharides on $\beta 1$ integrin. Cell lysates were either precipitated with agarose-bound L-PHA (left panel) or used directly for SDS-PAGE (right panel), followed by western blotting with anti- $\beta 1$ integrin antibody. IP, immunoprecipitation; M, mock; TB, GnT-VB; WB, western blotting.

is possible that GnT-VB expression functions in the proper timing of neurite extension to target particular innervations during nervous system development by modulating integrin function. Manganese-induced neurite outgrowth of PC12 cells has been known to be strictly mediated by integrin activation and requires the presence of ECM for the initiation of neurite outgrowth (Lin *et al.* 1993). Therefore, it is reasonable to conclude that enhanced neurite outgrowth by manganese treatment in PC12/GnT-VB cells demonstrates a modulation of $\beta 1$ integrin function by GnT-VB expression.

Changes in the *N*-linked glycosylation of $\beta 1$ integrin have been shown to affect its cell adhesion function. Akiyama *et al.* (1989) reported that inhibition of *N*-linked glycosylation maturation of $\alpha 5\beta 1$ integrin by 1-deoxymannojirimycin treatment (MNJ), an inhibitor of Golgi α -mannosidase IA/IB, renders integrin heterodimers unable to bind a fibronectin affinity column. Furthermore, inhibition of *N*-linked glycosylation by tunicamycin treatment blocked not only $\alpha 5\beta 1$ integrin pairing, but also the surface expression of functional $\alpha 5\beta 1$ integrin (Zheng *et al.* 1994), suggesting that *N*-linked glycosylation of $\beta 1$ integrin plays critical functions ranging from α - $\beta 1$ subunit dimerization to the surface expression of functional integrin receptors. PC12 cells express a number of integrin heterodimers, including a collagen receptor and a laminin receptor, $\alpha 1\beta 1$ and $\alpha 3\beta 1$ integrin, respectively (Lin *et al.* 1993; Zhang *et al.* 1993). The results in this study suggest that function of both receptors may be modulated by GnT-VB expression.

In studies also using PC12 cells by Taniguichi's laboratory, expression of GnT-III, a glycosyltransferase that synthesizes bisecting *N*-linked glycans, inhibited PC12 neurite outgrowth induced by either NGF or a combination of EGF/integrin signaling (Ihara *et al.* 1997; Partridge *et al.* 2004). Introduction of bisecting *N*-linked glycans on TrkA or the EGF receptor by GnT-III over-expression caused down-

regulation of growth factor-mediated signaling, resulting in inhibition of neurite outgrowth. In our study, no apparent changes in NGF-TrkA and downstream ERK activation signaling were observed, however, indicating that the mechanism of modulation of neurite outgrowth in PC12/GnT-VB appears to differ from that observed when GnT-III is expressed in PC12 cells. Thus, taken together, these results underscore that changes in *N*-linked glycan branching can regulate cell adhesion and neuritogenesis, albeit by more than one type of signaling pathway.

The effects of aberrant *N*-linked glycosylation of cell adhesion receptors on cell motility have been documented (Fernandes *et al.* 1991; Demetriou *et al.* 1995; Guo *et al.* 2002, 2003). In particular, $\beta 1$ integrin function was shown to be affected by increased levels of $\beta(1,6)$ branched *N*-linked glycans, as well as increased $\alpha(2,6)$ sialylation, that led to reduced integrin-dependent cell adhesion and increased cell migration on ECM ligands (Guo *et al.* 2002; Bellis 2004). The role of integrins in neurite outgrowth and axon guidance during nervous system development has only recently been characterized (Suter and Forscher 2000; Kiryushko *et al.* 2004; Nakamoto *et al.* 2004). The interaction of axon guidance proteins (e.g. netrin) with integrins (Yebra *et al.* 2003) suggests that the basic mechanism of directional migration of various cell types is, in fact, in many ways similar to the mechanism of neurite outgrowth in neuronal cell types. Regarding the role of integrin-ECM interactions in neuronal differentiation, Guan *et al.* (2003) demonstrated that sensory neurons differentiate and extend neurites for different target innervations, based on the types of integrin receptors expressed by the neurons and subsequent integrin-ECM interactions, rather than the types of neurotrophin receptor expressed. Furthermore, the authors also showed that the expression levels of various integrin subunits were transcriptionally regulated as neuronal differentiation progressed. Interestingly, while target preference of sensory neurons was dependent of integrin expression profiles, neurotrophin receptors expression did not show a significant influence both in the presence and absence of neurotrophins during target innervations. These results strongly support the concept that innervations of sensory neurons during nervous system development are substantially regulated by specific integrin-ECM interactions. These studies support a plausible hypothesis that altered glycosylation of integrin receptors can lead to a direct modulation of neuronal differentiation in an integrin-dependent manner. While integrin glycosylation would likely not affect a specific integrin-ECM preference during target innervations, it could, however, regulate the timing or the rate of neural network formations.

In summary, our results demonstrate that alteration of $\beta 1$ integrin function by GnT-VB causes $\beta 1$ integrin-dependent enhancement of neurite extension in both NGF- and manganese-induced neurite outgrowth on collagen and laminin substrates. Effects on signaling pathways other than

NGF-TrkA by GnT-VB expression, however, cannot be excluded at present. GnT-VB is an endogenous branching enzyme for *O*-linked mannosyl glycans, an important brain-specific glycan structure (Krusius *et al.* 1987; Inamori *et al.* 2004). The presence of *O*-linked mannosyl glycans has been shown to be necessary for α -dystroglycan/laminin-mediated neuronal cell adhesion to ECM. While it is not known precisely how the branching of *O*-mannosyl glycans affects their function in α -dystroglycans/laminin interaction, the HNK-1 epitope was found on *O*-linked mannosyl glycans with 2-mono- and 2,6-di-substituted mannose, suggesting a potential role for the branching of *O*-linked mannosyl glycans for the expression of this epitope (Yuen *et al.* 1997). Future studies will evaluate the effects of GnT-VB deficiency during mouse embryonic development to determine if GnT-VB expression contributes to normal nervous system development, and whether increased branching of *O*-mannosyl glycans by GnT-VB can modulate α -dystroglycans/laminin interaction. As it appears that GnT-V and GnT-VB can glycosylate an overlapping repertoire of glycan acceptors, one may be able to compensate for the absence of the other. Mice that lack expression of both enzymes may therefore be required to shed light on their functions in neuronal development.

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