Dependence of neurotrophic factor activation of Trk tyrosine kinase receptors on cellular sialidase

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A direct link between receptor glycosylation and activation following natural ligand interaction has not been observed. Here, we discover a membrane sialidase-controlling mechanism that depends on ligand binding to its receptor to induce enzyme activity which targets and desialylates the receptor and, consequently, causes the induction of receptor dimerization and activation. We also identify a specific sialyl α-2,3-linked β-galactosyl sugar residue of TrkA tyrosine kinase receptor, which is rapidly targeted and hydrolyzed by the sialidase. Trk-expressing cells and primary cortical neurons following stimulation with specific neurotrophic growth factors express a vigorous membrane sialidase activity. Neuraminidase inhibitors, Tamiflu, BCX1812, and BCX1827, block sialidase activity induced by nerve growth factor (NGF) in TrkA-PC12 cells and by brain-derived neurotrophic factor (BDNF) in primary cortical neurons. In contrast, the neuraminidase inhibitor, 2-deoxy-2,3-dehydro-α-D-galactosyl neuraminic acid, specific for plasma membrane ganglioside Neu3 and Neu2 sialidases has no inhibitory effect on NGF-induced pTrkA. The GM1 ganglioside specific cholera toxin subunit B applied to TrkA-PC12 cells has no inhibitory effect on NGF-induced sialidase activity. Neurite outgrowths induced by NGF-treated TrkA-PC12 and BDNF-treated PC12nnr5 stably transfected with TrkB receptors (TrkB-nnr5) cells are significantly inhibited by Tamiflu. Our results establish a novel mode of regulation of receptor activation by its natural ligand and define a new function for cellular sialidases.

Keywords: cell differentiation/cell signaling/receptor activation/sialic acid/TrkA tyrosine kinase receptor/trypanosome trans-sialidase/cellular sialidase

Introduction

Nucleic acids, proteins, and polysaccharides are the three major classes of natural molecules involved in signal transduction. Although the structure and functional roles of nucleic acids and proteins are well known, those of carbohydrates are much less understood. Carbohydrates are the most complex and diverse class of molecules, usually found as constituents of glycoconjugates (Werz and Seeberger 2005). Glycoconjugates can be glycoproteins or glycolipids, and are known to mediate inflammation, cell–cell recognition, immunological responses, metastasis, and fertilization (Varki 1993). For secreted and cell membrane-bound receptors, glycosylation status may be an important requirement for their translocation and function. For example, partial glycosylation is required for the processing and/or hormone-binding activity of insulin receptor (Ronnett et al., 1984), epidermal growth factor (EGF) receptor (Sliker and Lane 1985; Soderquist and Carpenter 1984), nicotinic acetylcholine receptor (Merlie and Smith 1986), and members of the G-protein-coupled class of receptors, such as the vasoactive intestinal peptide receptor (Merlie and Smith 1986), somatostatin receptor (Rens-Domiano and Reisine 1991) and β-adrenergic receptor (Cervantes-Oliver et al. 1988; Rand et al. 1990). However, a direct link between glycosylation and receptor activation following ligand interaction has not yet been observed.

Insight for the role of glycosylation in receptor activation came from the well-characterized model of TrkA family tyrosine-protein kinase receptors which function as signaling receptors for the neurotrophin family of molecules of nerve growth factor (NGF). We showed that a highly purified recombinant trans-sialidase (TS) derived from Trypanosoma cruzi parasites targets TrkA receptors on TrkA-expressing rat pheochromocytoma cell line PC12 cells and colocalizes with TrkA internalization and phosphorylation (pTrkA) (Woronowicz et al. 2004). TS has two types of catalytic activities (Colman and Smith 2002; Lee and Kim 2001): a dominant sialyltransferase activity catalyzing the transfer of sialic acid to terminal β-D-galactose acceptors, and a much lower neuraminidase activity that results in sialic acid release from sialyl-oligosaccharides. The sialidase activity but not the sialylltransferase of TS was involved in activating TrkA receptors. Similar findings were observed using a highly purified recombinant α-2,3-neuraminidase (Streptococcus pneumoniae) (Woronowicz et al. 2004). At the same time, Chuenkova and PereiraPerrin (2004) also provided evidence that T. cruzi parasites bind TrkA via their neuraminidase in a NGF-inhibitable manner, which leads to TrkA autophosphorylation, activation of the PI3K/Akt kinase pathway and, eventually, to cell survival and neurite outgrowth. Their
T. cruzi neuraminidase did not react with the neurotrophin receptor p75NTR (Chukenova and PereiraPerrin 2004).

When NGF binds with its receptor TrkA on a neuron, the commonly held theory is that the retrograde survival signals involve the ligand–receptor complexes to be internalized into vesicles (Bhattacharya et al. 1997; Grimes et al. 1996, 1997), and retrogradely transported to the cell bodies providing survival signals to the neuron (Bhattacharya et al. 1997; MacInnis and Campenot 2002; Riccio et al. 1997; Senger and Campenot 1997; Tsui-Pierchal and Ginty 1999; Watson et al. 1999a, 1999b). NGF is restricted to the synaptic area located far from the cell body where it is believed to exert its effect (Jullien et al. 2002). This theory has been challenged. Other studies show that neuronal survival signals can reach the cell bodies unaccompanied by the NGF that initiated it (MacInnis and Campenot 2002; Senger and Campenot 1997).

These retrograde survival signals do not depend on internalization and transport of NGF. These data led to the proposal that binding of NGF to TrkA receptors in nerve terminals results in the rapid propagation of a NGF-independent “wave” of TrkA receptor activation (Campenot and MacInnis 2004; MacInnis and Campenot 2002; Miller and Kaplan 2002). This concept is consistent with a recent finding of a ligand-independent wave of EGF receptor activation originating from EGF (Verber et al. 2000). Based on these findings, the precise mechanism of NGF-independent TrkA receptor activation is unclear (Campenot and MacInnis 2004; MacInnis and Campenot 2002; Miller and Kaplan 2002). Our findings show that NGF binding to TrkA induces one or more cellular sialidases that specifically target and hydrolyze sialyl α-2-3-linked β-galactosyl residues of TrkA, an initial step for receptor dimerization, internalization, and subsequent activation in Trk expressing cells and primary cortical neurons. This process is sufficient for the subsequent development of neurite outgrowth in these cells.

Results

Identification of a specific sialyl α-2,3-linked β-galactosyl residues of TrkA involved in receptor activation

If TrkA desialylation is required for receptor activation following NGF binding, then we should observe an initial desialylation of phosphorylated TrkA. We tested this assumption by examining the extent of sialylation of immunoprecipitated TrkA and pTrkA using Maackia amurensis lectin (MAL-2), reactive against sialic acid residues α-2,3-linked to galactosyl residues. The TrkA-PC12 cell line (overly expressing human TrkA receptors in the rat pheochromocytoma cell line PC12) (Hempstead et al. 1992) was used as a well-characterized model to examine this question. After no treatment or treatment of TrkA-PC12 cells with NGF or TS, cells were lysed and processed to immunoprecipitate pTrkA with anti-phospho Trk (pTyr490) antibody or nonactivated Trk with pan anti-Trk antibody followed by lectin blot analysis. MAL-2 reacted significantly less with the immunoprecipitated pTrkA when compared to the nonactivated Trk control (Figure 1). These results suggest that pTrkA had lost some sialyl α-2,3-linked β-galactosyl residues during NGF-induced activation consistent with the sialidase activity of the purified recombinant TS.

If cellular sialidase(s) is involved in the activation of TrkA, it should be possible to block its activity prior to NGF stimulation. TrkA-PC12 cells were pretreated with different lectins at various doses (0.1–100 μg/mL) for 30 min, washed and stimulated with 50 ng NGF/mL for 15 min. The cells were fixed, permeabilized, and immunostained with rabbit anti-pTyr490 of Trk followed with Alexa Fluor568 anti-rabbit IgG. Stained cells were visualized by epi-fluorescence microscopy. MAL-2 completely blocks NGF-induced pTrkA in a dose dependent manner (Figure 2A). In contrast, Sambucus nigra lectin (SNA which binds to α-2,6 sialic acid linked to terminal galactose and to lesser degree α-2,3 linkage), peanut agglutinin (PNA, galactosyl (β-1,3) N-acetylglucosamine), succinylated wheat germ agglutinin (sWGA, N-acetylglucosamine residues), soybean agglutinin (SBA, terminal α- or β-linked N-acetylgalactosamine), Ulex europaeus agglutinin I (UEA-I, α-linked fucose), Ricinus communis agglutinin I (RCA-1, galactose and N-acetylgalactosamine), and Dolichos biflorus agglutinin (DBA, α-linked N-acetylgalactosamine) do not block NGF-induced pTrkA expression. It is noteworthy that neither MAL-2 nor SNA treatment of TrkA-expressing cells blocks NGF binding to TrkA (Figure 2B). Together, these results strongly suggest that MAL-2 binding to TrkA-expressing cells at α-2,3 sialic acid linked to terminal galactose blocks NGF-induced cellular sialidase(s) activity from targeting the receptor, and so it can no longer hydrolyze these sialic acid residues and activate TrkA.

If NGF-induced pTrkA involves the hydrolysis of sialyl α-2,3-linked β-galactosyl residues, then MAL-2 binding to pTrkA should have a markedly reduced colocalization with pTrkA in NGF-stimulated TrkA-PC12 cells compared with nonactivated TrkA. To test this, TrkA-PC12 cells were treated with NGF for 15 min, TS or mutant TS with glutamic acid residue at position 98 replacing aspartic acid (TSΔAsp98-Glu) (Laroy and Contreras 2000; Woronowicz et al. 2004) for 60 min or were left untreated as controls. They were fixed, permeabilized, and immunostained with biotinylated MAL-2 and then with streptavidin and Alexa Fluor568 anti-rabbit IgG. Stained cells were visualized with a confocal inverted microscope (Leica TCS SP2 MP inverted Confocal Microscope). As shown in Figure 3A, MAL-2 binds to TrkA on TrkA-PC12 cells and colocalizes with nonactivated TrkA (71% overlay), but is significantly reduced with NGF-induced pTrkA (39% overlay) or TS-induced pTrkA (31% overlay). During the 60-min period, the catalytically inactive mutant TSΔAsp98-Glu does not hydrolyze α-2,3 linked sialic acids as revealed by 59% overlay of MAL-2 colocalization with nonactivated TrkA. These latter results are consistent with our previous findings that the catalytically inactive mutant TSΔAsp98-Glu does not induce pTrkA, 60 min after the treatment of TrkA-PC12 cells (Woronowicz et al. 2004). Thus, it appears that NGF-induced pTrkA receptors have fewer sialyl α-2,3-linked β-galactosyl residues compared with nonactivated TrkA.

To determine the specificity of the NGF-induced cellular sialidase(s) activity in TrkA-expressing cells, we asked if the cellular sialidase(s) specifically targets and hydrolyzes sialyl
α-2,3-linked β-galactosyl residues on TrkA receptors. To test this, we hypothesized that MAL-2 binding to sialyl α-2,3-linked β-galactosyl residues on TrkA-expressing cells would not colocalize with NGF-induced pTrkA. The rationale for this hypothesis stems from the data in Figure 2A. In contrast, SNA binding to sialyl α-2,6-linked β-galactosyl residues on TrkA-expressing cells would colocalize with pTrkA. Here, TrkA-PC12 cells were pretreated with either biotinylated SNA or biotinylated MAL-2 at 100 μg/mL for 30 min, washed and then stimulated with 50 ng/mL NGF for 15 min. The cells were fixed, permeabilized, and immunostained with rabbit anti-pTyr490 of Trk followed simultaneously with Alexa Fluor568 anti-rabbit IgG and streptavidin-fluorescein. The overlay images are from confocal microscopy. As shown in Figure 3B, SNA binds to TrkA receptors on TrkA-expressing PC12 cells and colocalizes with.
NGF-induced pTrkA. In contrast, MAL-2 lectin also binds to TrkA-PC12 cells with hardly any colocalization with pTrkA. These results indicate that SNA bound to sialyl α-2,3-linked β-galactosyl residues does not block TrkA activation, whereas MAL-2 bound to sialyl α-2,3-linked β-galactosyl residues blocks pTrkA. These observations suggest that sialyl-α-2,3-linked β-galactosyl residues are targeted and hydrolyzed during NGF-induced activation of TrkA, whereas sialyl α-2,6-linked β-galactosyl residues are spared in this process.

**NGF binding to TrkA receptors induces cellular sialidase(s)**

To confirm that NGF binding to its TrkA receptor induces cellular sialidase(s), TrkA-PC12 cells were pretreated with purified neuraminidase inhibitors, oseltamivir phosphate (Tamiflu), DANA, BCX-1812, and BCX-1827 at different concentrations (0.1–100 µM) for 2 h and then stimulated with 50 ng/mL NGF for 15 min. Cells were fixed, permeabilized, and immunostained with goat anti-pTyr490 Trk followed by Alexa Fluor568 rabbit anti-goat IgG. The images were obtained from epi-fluorescence microscopy using a 40× objective. Quantitative analysis was done by assessing the density of cell staining corrected for background in each panel for MAL-2 and SNA using Corel Photo Paint 8.0 software. Each bar in the figures represents the mean corrected density of staining ± SEM for all cells within the respective images. The data are a representation of one out of two experiments showing identical results.

**(A)** MAL-2 binding to sialyl α-2,3-linked β-galactosyl residues of TrkA receptors blocks NGF-induced TrkA phosphorylation (pTrkA). TrkA-PC12 cells were pretreated with different lectins at indicated concentrations (0.1–100 µg/mL) for 30 min, washed and stimulated with 50 ng/mL NGF for 15 min. Cells were fixed, permeabilized, and immunostained with polyclonal goat anti-pTyr490 Trk followed by Alexa Fluor568 rabbit anti-goat IgG. The images were obtained from epi-fluorescence microscopy using a 40× objective. Quantitative analysis was done by assessing the density of cell staining corrected for background in each panel for MAL-2 and SNA using Corel Photo Paint 8.0 software. Each bar in the figures represents the mean corrected density of staining ± SEM for all cells within the respective images. The data are a representation of one out of two experiments showing identical results.

**Fig. 2.**

**(A)** MAL-2 binding to sialyl α-2,3-linked β-galactosyl residues of TrkA receptors blocks NGF-induced TrkA phosphorylation (pTrkA). TrkA-PC12 cells were pretreated with different lectins at indicated concentrations (0.1–100 µg/mL) for 30 min, washed and stimulated with 50 ng/mL NGF for 15 min. Cells were fixed, permeabilized, and immunostained with polyclonal goat anti-pTyr490 Trk followed by Alexa Fluor568 rabbit anti-goat IgG. The images were obtained from epi-fluorescence microscopy using a 40× objective. Quantitative analysis was done by assessing the density of cell staining corrected for background in each panel for MAL-2 and SNA using Corel Photo Paint 8.0 software. Each bar in the figures represents the mean corrected density of staining ± SEM for all cells within the respective images. The data are a representation of one out of two experiments showing identical results.

**(B)** Biotinylated NGF binds to TrkA-PC12 cells pretreated with MAL-2. TrkA-PC12 cells were pretreated with MAL-2 or SNA at 100 µg/mL for 30 min, washed, and stimulated with 50 ng/mL biotinylated NGF for 15 min. Cells were fixed, permeabilized, and immunostained with FITC-streptavidin. The images were obtained from epi-fluorescence microscopy using a 40× objective. Quantitative analysis was done as described in panel A. Each bar in the figures represents the mean corrected density of staining ± SEM for all cells within the respective images. The data are a representation of one out of three experiments showing identical results.
neuraminidases (Chavas et al. 2005). Together, the DANA results shown in Fig. 4 suggest that NGF-induced cellular sialidase activity is not Neu2 sialidase.

To further investigate the inhibitory effect of Tamiflu on NGF-induced pTrkA expression in TrkA-PC12 cells, cells pretreated with 400–500 μM Tamiflu for 30 min followed by 50 ng/mL NGF or left untreated as controls were analyzed for pTrkA expression by flow cytometry (FACS) and Western blot. Cells were fixed, permeabilized, and stained with biotinylated-MAL-2 for 30 min. After extensive washing, cells were immunostained with rabbit anti-Trk or rabbit anti-pTyr490 of Trk as indicated followed simultaneously with fluorescein (FITC) conjugated streptavidin (green) and Alexa Fluor568 anti-rabbit IgG (red). The images were visualized with a confocal inverted microscope (Leica TCS SP2 MP inverted Confocal Microscope) using a 100× objective (oil). Images were captured using a z-stage of 8–10 images per cell at 0.5-mm steps. To calculate the amount of colocalization (yellow) in the selected images, the Pearson correlation coefficient was measured and expressed as a percentage using Image Pro Plus software. Each bar in the figure represents the mean Pearson correlation coefficient ± SEM for four independent experiments (n). The images are a representation of one out of four (n) independent experiments showing similar results. Significant differences at 95% confidence using the Dunnett multiple comparison test was determined compared to the MAL-2 and TrkA group.

(B) Both MAL-2 and SNA bind to TrkA receptors on NGF-stimulated TrkA-PC12 cells but only SNA colocalizes pTrkA. TrkA-PC12 cells were pretreated with biotinylated lectins at indicated concentrations for 30 min, washed and then stimulated with 50 ng/mL NGF for 15 min. Cells were fixed, permeabilized, and immunostained with rabbit anti-pTyr490 of Trk and followed simultaneously with Alexa Fluor568 anti-rabbit IgG (red) and streptavidin-FITC (green). Cells were visualized using a confocal inverted microscope with a 100× objective (oil). Images were captured using a z-stage of 8–10 images per cell at 0.5-mm steps and were processed and merged using LCS Lite software. Each bar in the figure represents the mean amplitude ± SEM of lectin binding or pTrkA expression for all cells (n) within the respective images. The indicated significant differences at 95% confidence using the Dunnett multiple comparison test are compared to NGF group. The data are a representation of one out of three experiments showing similar results.

Fig. 3. (A) MAL-2 colocalizes with TrkA but minimally with pTrkA. TrkA-PC12 cells were grown in 24-well tissue culture plates on 12-mm circular glass slides coated with poly-α-lysine for 24 h prior to stimulation with 50 ng/mL NGF (15 min), 200 ng/mL TS (60 min), 200 ng/mL mutant TSDAsp98-Glu (60 min) or were left untreated. Cells were fixed, permeabilized and stained with biotinylated-MAL-2 for 30 min. After extensive washing, cells were immunostained with rabbit anti-Trk or rabbit anti-pTyr490 of Trk as indicated followed simultaneously with fluorescein (FITC) conjugated streptavidin (green) and Alexa Fluor568 anti-rabbit IgG (red). The images were visualized with a confocal inverted microscope (Leica TCS SP2 MP inverted Confocal Microscope) using a 100× objective (oil). Images were captured using a z-stage of 8–10 images per cell at 0.5-mm steps. To calculate the amount of colocalization (yellow) in the selected images, the Pearson correlation coefficient was measured and expressed as a percentage using Image Pro Plus software. Each bar in the figure represents the mean Pearson correlation coefficient ± SEM for four independent experiments (n). The images are a representation of one out of four (n) independent experiments showing similar results. Significant differences at 95% confidence using the Dunnett multiple comparison test was determined compared to the MAL-2 and TrkA group.
NGF-treated TrkA-PC12 and brain-derived neurotrophic factor (BDNF) treated TrkB-nnr5 cells following pretreatment with Tamiflu. When the cells were stimulated with 100 ng/mL NGF or 100 ng/mL BDNF for 3 days in culture in the absence or presence of 200 or 500 μM Tamiflu, the cells extended neurites but these were significantly inhibited with Tamiflu (Figure 6). These findings suggest that Tamiflu inhibition of cellular sialidase induced by NGF- or BDNF-stimulated Trk expressing cells not only blocks Trk phosphorylation but also prevents neurotrophic factor induced cell differentiation (neurite outgrowth).

To advance our hypothesis that NGF binding to TrkA induces cellular sialidase activity, an assay to detect sialidase activity in viable cells was needed. TrkA-PC12 cells were grown on 12-mm circular glass slides in medium containing 5% horse serum and 4% fetal calf serum, and treated with 50 ng/mL NGF for 15 min. Cells were fixed, permeabilized, and immunostained with goat anti-pTyr490 TrkA followed with Alexa Fluor568 rabbit anti-goat IgG. Stained cells were visualized by epi-fluorescence microscopy using a 40× objective. Quantitative analysis was done by assessing the density of cell staining corrected for background in each panel using Corel Photo Paint 8.0 software. Each bar in the figures represents the mean corrected density of staining ± SEM for all cells (n) within the respective images. Asterisk (*) represents significant differences at 95% confidence using the Dunnett multiple comparison test compared to control (Ctrl) in each group. The data are a representation of one out of three experiments showing similar results. (B) Western blots of DANA treatment of TrkA-PC12 cells on NGF- and TS-induced pTrkA expression. Cells were grown in 25-cm² flasks at 90% confluence. They were pretreated with 500 μM Tamiflu in serum free medium for 24 h or left untreated as control. Cells were stimulated with 50 ng/mL NGF for 15 min or 200 ng/mL wt TS and immediately lysed in 500 μL of 1× SDS sample buffer. To each cell lysate was added 3% 2-mercaptoethanol and boiled for 10 min. Cell lysates were resolved by 8% SDS-PAGE gel, and the blots probed with polyclonal goat anti-pY490 Trk antibody followed by HRP conjugated secondary anti-goat IgG antibody, and Western Lightning Chemiluminescence Reagent Plus. Sample concentration for gel loading was determined by BioRad reagent.
and 200 μM Tamiflu. As shown in Figure 7A, NGF treatment of TrkA-PC12 cells induces sialidase activity within 6 min as revealed by a halo of fluorescence (blue color) surrounding the cells caused by the emission of free 4-methylumbelliferoine hydrolyzed from 4-MUNANA substrate compared with untreated control cells. Tamiflu completely blocks NGF-induced cellular sialidase activity in these cells. When TrkA-expressing cells were pretreated with MAL-2 and followed by NGF and 4-MUNANA addition, a fluorescence halo also arose suggesting that NGF induces cellular sialidase activity even in the presence of MAL-2 (Figure 7B). Cells treated with MAL-2 alone do not express sialidase activity. These data indicate again that MAL-2 does not block NGF binding to its receptor, and NGF binding to its receptor induces cellular sialidase(s) activation on the cell surface even in the presence of MAL-2. If the TrkA receptor is critical in the process of activating sialidase(s), then we should not expect to see fluorescence in NGF-treated TrkA-deficient a tyrosine kinase deficient mutant (PC12<sup>nnr5</sup>) cells. The data in Figure 7C clearly show this to be the case. We also questioned whether or not the fluorescence surrounding NGF-treated TrkA-expressing cells is due to secreted sialidase(s). Medium was taken from the reaction wells containing the viable cells and added immediately to 4-MUNANA substrate. A positive control sialidase (Clostridium perfringens) which has a specific activity of 1 U per 1 μmol of N-acetylleucosaminic acid per minute was also added to the 4-MUNANA substrate. As shown in Figure 7D, the fluorescence surrounding the NGF-treated cells seen in Figure 7A is not due to a form of secreted or shed sialidase from the cells. Since plasma membrane sialidase Neu3 is specific for GM1 gangliosides (Duchemin et al. 2002; Kimura et al. 2001; Rabin and Mocchetti 1995), we asked whether Neu3 sialidase could be the key player in our NGF-induced sialidase activity. To test this, GM1 specific cholera toxin subunit B (CTX-B) was added to TrkA-PC12 cells in the

![Fig. 5. Flow cytometry analysis of Tamiflu inhibition of NGF-induced pTrkA expression in TrkA-PC12 cells. (A) Cells grown in 25-cm<sup>2</sup> flasks at 90% confluence were treated with 500 μM Tamiflu in serum free medium for 24 h or left untreated. They were stimulated with 50 ng/mL NGF for 15 min and immediately fixed, permeabilized, and immunostained with FITC conjugated IgG anti-pY490 Trk antibody. Cells were analyzed by Beckman Coulter Epics XL-MCL flow cytometry and Expo32 ADC software (Beckman Coulter). Overlay histograms are displayed. Untreated control cells are represented by the black-filled histogram. Cells pretreated with Tamiflu and stimulated with NGF are depicted by the gray-filled histogram. Cells treated with NGF are depicted by the unfilled histogram with the black line. The mean fluorescence for each histogram is indicated for 7000 acquired cells (98% gated). The data are a representation of one out of three experiments showing similar results. (B) Western blot analysis of Tamiflu inhibition of NGF-induced pTrkA expression. Cells were grown in 75-cm<sup>2</sup> flasks at 90% confluence. They were pretreated with 500 μM Tamiflu in serum free medium for 24 h or left untreated. Cells were stimulated with 50 ng/mL NGF for 15 min and immediately lysed in 500 μL of 1× SDS sample buffer. To each cell lysate was added 3% 2-mercaptoethanol and boiled for 10 min. Cell lysates were resolved by 8% SDS-PAGE gel, and the blots probed with polyclonal goat anti-pY490 Trk antibody followed by HRP conjugated secondary anti-goat IgG antibody, and Western Lightning Chemiluminescence Reagent Plus. Sample concentration for gel loading was determined by BioRad reagent. Quantitative analysis was done by assessing the density of 140-kDa band corrected for background pixels in each lane using Corel Photo Paint 8.0 software. Each bar in the figure represents the mean corrected density of band ± SEM for five replicate measurements (n = 5). Significant differences at 95% confidence was done using the Dunnett multiple comparison test compared to NGF group. The data are a representation of one out of four experiments showing similar results.

![Fig. 6. Tamiflu inhibits neurite outgrowth in NGF-stimulated TrkA-PC12 cells and BDNF-stimulated TrkB-nnr5 cells. Cells were grown on 12-mm circular glass slides precoated with poly-l-lysine either in medium containing 5% horse serum and 3% FBS for 3 days (Ctrl), in medium containing 100 ng/mL of NGF for TrkA-PC12 cells or 100 ng/mL BDNF for TrkB-nnr5 cells, or in combination with 200–500 μM Tamiflu and NGF or BDNF with respective cells. Cells were fixed, permeabilized, and stained with phalloidin-rhodamine. Cell staining was analyzed qualitatively using epi-fluorescence microscopy and quantitatively by counting the number of cells with multiple neurite extensions (>200 of the total cells; one or more extensions greater than 2 cell bodies in length). The data are a representation of one out of three experiments showing identical results. Significant differences at 95% confidence was done using the Dunnett multiple comparison test compared to ligand in each group.]
presence of 4-MUNANA substrate and NGF does not block NGF-induced sialidase activity (Figure 7E). In addition, DANA added to the cells in the presence of 4-MUNANA and NGF does not block NGF-induced sialidase activity (Figure 7E). These latter data suggest that plasma membrane Neu3 and cytosolic Neu2 sialidases may not be the key players in NGF-induced sialidase activity.

We also tested whether NGF-induced sialidase activity can be observed in the cell suspensions of TrkA-PC12 cells. To 100 μL of cell suspension (3–4 × 10⁶ cells/mL) from 95% cell confluence in 25-cm² flasks of serum-free medium, 50 ng/mL NGF was added at different time intervals or was left untreated as controls. The NGF-treated and untreated cells (3 × 10⁵ cells) were immediately added to substrate buffer (20 mM Tris and 30 mM NaCl) containing aprotinin, leupeptin, phenylmethylsulphonylfluoride (PMSF) and 0.05 mM 4-MUNANA in cuvets with or without the presence of 200 μg/mL of either Tamiflu, BCX-1812 or BCX-1827. The fluorescence intensity readings are immediately taken over 15 min using the Varian Cary Eclipse Fluorescence Spectrophotometer at low and medium PMT voltage settings using fluorescence emission at 450 nm and an excitation at 365 nm. The sialidase activity in the cell samples (3 × 10⁵ cells) was calculated from a standard sialidase activity curve and expressed as nano Units (nU) per mg of 4-MUNANA substrate. Each bar in Figure 8A represents the mean ± SEM of the sialidase activity corrected for the background endogenous sialidase activity in untreated cells from three independent experiments. The data clearly show that NGF induces sialidase activity in TrkA-PC12 cells within 15 s, and this activity is blocked by neuraminidase inhibitors, Tamiflu, BCX-1812, and BCX-1827 (Figure 8B). It is noteworthy that the rapid sialidase activity in the cell suspensions following NGF treatment suggests an early sialidase activity followed by a subsequent expression on the cell membrane (Figure 7A).

**Primary cortical neurons express membrane sialidase activity following BDNF stimulation**

We also tested whether BDNF-induced sialidase activity could be observed in primary cortical neurons. Neurons were grown on 12-mm circular glass slides precoated with...
poly-d-lysine for 4 days in neurobasal medium supplemented with B27 and penicillin–streptomycin (Brewer 1995). After removing medium, 2.04 mM 4-MUNANA (4-MU) substrate [2'-4-(methylumbelliferyl)-α-D-N-acetylneuraminic acid] (Sigma) in Tris buffered saline pH 7.4 was added to each well alone (Ctrl), with 200 ng/mL BDNF or in combination with 200 ng/mL BDNF and 400 μM Tamiflu. The data are a representation of one out of three experiments showing identical results.

Fig. 9. (A) BDNF stimulated primary cortical neurons express membrane sialidase activity which is blocked by Tamiflu. Primary cortical neurons were grown on 12-mm circular glass slides precoated with poly-d-lysine for 4 days in neurobasal medium supplemented with B27 and penicillin–streptomycin (100 IU/ml). After removing medium, 2.04 mM 4-MUNANA (4-MU) substrate was added to cell monolayer in 500 μl of serum-free medium.

Discussion

The data presented here are the first observations that make the direct link between receptor glycosylation and activation of signal transducing receptors like Trk. These findings suggest a novel mechanism for Trk activation by neurotrophic growth factor binding. It is known that when NGF binds to TrkA, the subsequent signaling events appear to involve trafficking of the receptor into endosomes (Barker et al. 2002; Beattie et al. 2002; Butowt and von Bartheld 2001; Hauke 2002; Jullien et al. 2002; Shao et al. 2002; Whitmarsh and...
Several mammalian sialidases have been cloned and purified and classified according to their subcellular localization (Miyagi et al. 1990). They are lysosomal (Neu1), cytosolic (Neu2), and plasma membrane bound (Neu3). A new sialidase named Neu4 was cloned from murine brain and has similarities to Neu3 (Comelli et al. 2003). There is evidence for a human sialidase (Neu4) localized to the mitochondrial (Yamaguchi et al. 2005) or lysosomal lumen (Seyran et al. 2003). Plasma membrane sialidase (Neu3) appears to specifically hydrolyze GM1 gangliosides (Papini et al. 2004; Rodriguez et al. 2001; Sasaki et al. 2003). Lysosomal sialidase (Neu1) is a glycoprotein enzyme that is only active as a part of a molecular multi-enzyme complex that contains β-galactosidase and cathepsin A (Lukong et al. 2000). Recent observations revealed that the intracellular distribution of sialidase encoded by the Neu1 gene is regulated by the signal sequence at the cytoplasmic tail, and that the sialidase can be detected within the lysosome matrix as well as in the plasma membrane under conditions of cell stimulation (Lukong et al. 2001). More recent data have indicated that the lysosomal carboxypeptidase, cathepsin A, which forms a complex with and activates Neu1 in the lysosome, is sorted to the plasma membrane of the differentiating monocyte cells similarly to Neu1. Both proteins are first targeted to the lysosome and then sorted to the LAMP-2-negative, MHC II positive vesicles, which later merge with the plasma membrane (Liang et al. 2006). Among the sialidases, the cytosolic form Neu2 normally shows the lowest expression level. The cytosolic sialidase is highly expressed in skeletal muscle (Fanzani et al. 2003; Sato and Miyagi 1996) as well as in the liver (Miyagi et al. 1996; Sato and Miyagi 1996) and elsewhere. The cytosolic form Neu2 in the differentiation of PC12 cells has been reported in its free form and in complex with the neuraminidase inhibitor, 2-deoxy-2,3-dehydro-α-N-acetylmuramic acid (DANA) (Chavas et al. 2005). Interaction between Neu2 and DANA showed similarities with bacterial and viral counterparts but also exhibited some differences in the active site arrangement and dynamic nature of the loops containing residues responsible for catalysis and substrate recognition. For the plasma membrane sialidase Neu3, it has been shown that Neu3 receptor needs to interact with GM1 ganglioside in order to dimerize, get phosphorylated, and trigger its signaling pathway (Duchemin et al. 2002; Kimura et al. 2001; Rabin and Mochetti 1995). GM1 is the product of Neu3 that, additionally, is the true membrane sialidase in plasma membrane (Kopitz et al. 1998; Miyagi and Tsuiki 1986). In fact, the neuraminidase inhibitor, oseltamivir has been found to block GM1 ganglioside-regulated excitatory in opioid receptor-mediated hyperalgesia (Crain and Shen 2004). Because plasma membrane sialidase Neu3 is specific for GM1 gangliosides, this Neu3 sialidase could be involved in our NGF-induced sialidase activity. It is intriguing to speculate that Neu3 does not directly act on Trk sialic acid but, nevertheless, could be activated upon NGF binding, which would in turn produce GM1, allowing TrkA dimerization and eventually play a role in the further activation of other sialidases that would target Trk sialic acid. Actually, da Silva et al. (2005) have recently demonstrated that over-expression of plasma membrane ganglioside sialidase Neu3 (PMGS) in primary neurons produces a major increase in phosphorylated TrkA (pTrkA), which was further enhanced by NGF. In fact, pTrkA was precipitated with GM1 when PMGS was over-expressed, and the PMGS was shown to interact with pTrkA, especially after NGF stimulation (da Silva et al. 2005). These results indicate that PMGS could lead to localized activity of Trk receptors but their effects may be directed only to pTrkA. Other studies have shown that Neuro-2a and NG108-15 cells grown in the presence of a neuraminidase, an enzyme that increases the cell surface content of GM1, caused neurite outgrowth which, in the case of Neuro-2a, could be blocked by the GM1 specific cholera toxin subunit B (CTX-B). However, CTX-B agent applied to NG108-15 cells had no inhibitory effects (Fang et al. 2000). Since DANA (2-deoxy-2,3-dehydro-α-N-acetylmuramic acid) is an inhibitor specific for PMGS (da Silva et al. 2005) and cytosolic sialidase Neu2 (Chavas et al. 2005) as well as CTX-B blocks GM1 ganglioside from Neu3 interaction, both DANA and CTX-B did not have any inhibitory effect on our NGF-induced sialidase activity in TrkA-PC12 cells (in Figures 4 and 7E). Taken together, we speculate that the candidate cellular sialidase in our studies may be lysosomal sialidase Neu1 (Liang et al. 2006) or mitochondrial/lysosomal lumen Neu4 (Seyran et al. 2004; Yamasaki et al. 2005).

When TrkA-PC12 cells were pretreated with MAL-2 lectin, NGF-induced pTrkA was completely inhibited in a dose dependent manner (Figure 2A). MAL-2 binding to sialyl α-2,3-linked β-galactosyl residues on TrkA does not block NGF binding to TrkA. These results suggest that NGF-induced cellular sialidase activity targets sialyl α-2,3-linked β-galactosyl residues of TrkA distant from NGF binding residues. For the Trk receptors, deletion and mutagenesis analyses of the ectodomains have identified a necessary and sufficient ligand-binding fragment as well as a binding domain important for affinity and specificity (Arevalo et al. 2000; Arevalo et al. 2001; Ulltsch et al. 1999). Furthermore, the crystal structure of the complex between NGF and the ligand-binding domain of TrkA clearly defines the orientation of NGF with respect to the membrane and elucidates specificity and binding for the entire family (see review (Wiesmann and de Vos 2001)). Of the five domains comprising its extracellular portion, the immunoglobulin-like domain
proximal to the membrane (TrkA-d5 domain) is necessary and sufficient for NGF binding (Wiesmann et al. 1999). The structure is consistent with results from mutagenesis experiments for all neurotrophins. In fact, Arevalo et al. (2000), using different mutants of the TrkA extracellular regions, found that elimination of the first or second Ig-like domain leads to activation of the receptor. These results suggested a role for both Ig-like domains in the stabilization of the monomeric form of the receptor, perhaps, through repulsion that can be negated by ligand binding. The receptor activation caused by deletion of the first Ig-like domain was weak compared to that caused by deleting the second domain. The second Ig-like domain 5 appears to be more critical in preventing spontaneous receptor dimerization, and this correlates with the more important role played by this domain in NGF binding (Arevalo et al. 2000; Wiesmann et al. 1999). Although the two Ig-like ligand-binding domains of TrkA might be involved in blocking receptor homodimerization in the absence of ligand, it is unclear if and how the intracellular portions of Trks interact with each other.

In this report, we identify a specific Trk desialylation by membrane sialidase(s), which is induced by neurotropic factor binding to Trk receptor. We propose that NGF-induced sialidase activity may be targeting the second Ig-like domain 5. In fact, TrkA residues that are in contact with NGF in the NGF- TrkA-d5 complex have been sequenced (Wiesmann and de Vos 2001). There are three N-linked asparagine residues that are in contact with NGF but a total of six residues that might be available for sialidase interaction. Ligand binding to its receptor activates membrane bound sialidase(s) that may specifically target and hydrolyze one or more of these six N-linked sialyl α2,3,linked β-galactosyl residues of TrkA-d5 ectodomain. This subsequent sugar removal would facilitate receptor homodimerization, internalization, and activation. Indeed, we found that primary cortical neurons following stimulation with specific neurotrophic growth factors also express a vigorous membrane sialidase activity. Neuraminidase inhibitor, Tamiflu, blocks sialidase activity induced by BDNF in primary cortical neurons. Neurite outgrowths produced by NGF-treated TrkA-PC12 and BDNF-treated TrkB-nnr5 cells are also significantly inhibited by Tamiflu. The results in this report establish for the first time a novel mode of regulation of receptor activation by its natural ligand and also define a new function for cellular sialidase.

**Material and methods**

**Recombinant TS**

The recombinant TS and the mutant TSΔAsp98-Glu used in these experiments were isolated using the methylotrophic yeast *Pichia pastoris* system, and further purified using an affinity anti-E-tag antibody column (Laroy and Contreras 2000). Both the wild-type TS and mutant TSΔAsp98-Glu had the E-tag (pCAGGS-TSE) sequence linked to the C-terminus domain (Laroy and Contreras 2000). The mutant TSΔAsp98-Glu has a point mutation in the catalytic domain of TS reducing the sialidase and sialyltransferase activities to approximately 3.6% and 4.5%, respectively, of the wild type TS (Woronowicz et al. 2004). The sialidase activity of the enzymes was measured in Tris buffered Saline (TBS) pH 7.6 containing 0.2 mM 4-methylumbelliferyl-N-acetylneuraminic acid (MUNANA) at a temperature of 25 °C. After 15 min incubation, the reaction was stopped and the fluorescence of free 4-methylumbelliferone was measured with a CYTOFLUOR® Multi-Well Plate Reader Series 4000 (PerSeptive Biosystems, Brussels, Belgium). The sialyltransferase activity of the wild type TS and the mutant TSΔAsp98-Glu was measured in HEPES buffer pH 7.2 containing 20 μM N-acetylneuraminyl lactose and 220 mM APTS-labeled NA2FB sugar structures (asialo-, galactosylated biantennary, core-substituted with fucose and with bisecting GlcNAc). The NA2FB sugar structures were labeled and purified according to Callewaert et al. (Callewaert et al. 2001). For the analysis of the glycan structures, DSA-FACE method was used as previously described (Callewaert et al. 2001). The optimal dose of 200 ng TS or mutant TS per ml was previously determined (Woronowicz et al. 2004).

**Neurotrophic growth factors and neuraminidase inhibitors**

NGF (50 ng/mL) and BDNF (100 ng/mL) (Sigma, St. Louis, MO) were used at predetermined optimal dosage. Tamiflu (pure oseltamivir phosphate, Hoffmann-La Roche Ltd., Mississauga, Ontario, Lot no. BS00060168), BCX-1812 or BCX-1827 (BioCryst Pharmaceuticals Inc., Birmingham, AL), DANA (2-deoxy-2,3-dehydro-α-N-acetylenuraminic acid) (Sigma) and cholera toxin subunit B (CTX-B) (Sigma) were used at indicated concentrations.

**Lectins**

*M. amurenensis* lectin 2 (MAL-2) (Vector Laboratories Inc., Burlington, Ontario, Canada) binds specifically to α2,3 sialic acid linked to terminal galactose. *S. nigra* lectin (SNA which binds to α2,6 sialic acid linked to terminal galactose and to lesser degree α2,3 linkage), peanut agglutinin (PNA, galactosyl (β-1,3) N-acetylgalactosamine), succinylated wheat germ agglutinin (SWGA, N-acetylgalactosamine residues), soybean agglutinin (SBA, terminal α- or β-linked N-acetylgalactosamine), *U. europaeus* agglutinin I (UEA-I, α-linked fucose), *R. communis* agglutinin I (RCA-1, galactose, and N-acetylgalactosamine), and *D. biflorus* (horse gram) agglutinin (DBA, α-linked N-acetylgalactosamine) were used in these studies.

**Cell lines**

The NGF responsive PC12 rat pheochromocytoma cell line, PC12nnr5 (tyrosine kinase deficient mutant, NGF nonresponsive) (Kaplan and Miller 1997; Kaplan and Miller 2000), and TrkA-PC12 cell line (overly expressing human TrkA receptors) (Hempstead et al. 1992), were used in these studies as previously described (Woronowicz et al. 2004). The TrkB-nnr5 cells are PC12nnr cells stably transfected to express rat TrkB receptors (kindly provided by Dr Susan Meakin, Laboratory of Neural Signaling, Cell Biology Group, Robarts Research Institute, London, Ontario, Canada) (Baskey et al. 2002). In the presence of BDNF, the TrkB-nnr5 cells respond by stopping division and extending neurites, which is similar to the activity of NGF on TrkA-PC12 cells. All cell lines were grown at 37 °C in 5% CO₂ in culture media containing DMEM (Gibco Rockville, MD) supplemented with 5% horse serum (Gibco) and 3% fetal calf serum (Cansera International Inc., Rexdale, Ontario, Canada).
Primary cortical neurons

Primary cultures of mouse cortical neurons were prepared as described previously (Brewer 1995; Brewer 1997). Pregnant CD-1 mice were sacrificed on gestational day 17, and the fetuses immediately removed from the uterus. The cortex from these fetuses was collected and digested with trypsin (0.1%) and DNAse I (50 μg/mL). The tissues were pooled in sterile neurobasal medium supplemented with 2% B27 and penicillin–streptomycin (100 IU/mL) (Invitrogen Canada Inc., Life Technologies, Burlington, Ontario), triturated and filtered through 37 μm nylon mesh. The filtrate was centrifuged for 10 min at 1000 rpm and re-suspended in neurobasal medium at a concentration of 3 × 10^6 cells/mL. Cells were plated onto 24 well-plates containing 12-mm round coverslips precoated with poly-D-lysine (50 μg/mL), incubated in 5% CO₂ atmosphere at 37 °C and grown for 4 days before testing. The cultures consist of 95% neurons as determined by immunocytochemical examination using MAP-2 antibody.

Lectin blot of TrkA and phosphorylated Trk (pTyr490)

TrkA-PC12 cell line were grown at 37 °C in 5% CO₂ in culture media. The cells were stimulated with either 50 ng/mL NGF for 15 min, 200 ng/mL TS for 60 min or left untreated as controls (Ctrl) (see Figure 1). Cells were lysed and processed to immunoprecipitate phosphorylated TrkA (pTrkA) with affinity purified polyclonal rabbit anti-pTyr490 antibody (Abcam Ltd., Cambridgeshire, UK) or nonactivated TrkA (Ctrl) with pan anti-TrkA antibody (kind gift from Dr Gregory Ross, Queen’s University). The immunoprecipitated TrkA receptors were resolved by 8% SDS-PAGE gel followed by either MAL-2 lectin blot analysis, anti-pTrkA or anti-pan Trk antibodies. The blots were probed with biontinated M. amurenensis lectin 2 (MAL-2; Vector Laboratories Inc., Burlington, Ontario, Canada), followed by horseradish peroxidase (HRP)-conjugated streptavidin and Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer, Boston, MA). To determine protein loading, the gel was stained with Coomassie Brilliant Blue (Sigma). Quantitative analysis was done by assessing the density of gel bands after background correction using Corel Photo Paint 8.0 software.

Lectin colocalization with pTrkA and TrkA

TrkA-PC12 cells were grown in 24-well tissue culture plates on 12-mm circular glass slides coated with poly-D-lysine (Sigma) for 24 h prior to experimental treatment. After 2–3 days of treatment, the cells were fixed in 3.7% paraformaldehyde for 30 min, permeabilized with 0.2% Triton X-100 in TBS on ice for 5 min followed with a blocking solution of 3% bovine serum albumin on ice for 20 min. The cells were stained with phalloidin-rhodamine (Sigma) for 1 h at 37 °C. Cell staining was analyzed qualitatively using epi-fluorescence microscopy and quantitatively by counting the number of cells with multiple neurite extensions (≥200 of the total cells; one or more extensions greater than 2 cell bodies in length).

Flow cytometry

Cells grown in 25-cm² flasks at 90% confluence were treated with 500 μM Tamiflu in serum free medium for 24 h or left untreated. They were stimulated with 50 ng/mL NGF for 15 min and immediately fixed, permeabilized, and immunostained with fluorescein conjugated IgG goat anti-pY490 Trk antibody. 7000 cells were acquired on a Beckman Coulter (Miami, FL) Epics XL-MCL flow cytometer and analyzed with Expo32 ADC software (Beckman Coulter). For overlay histograms, untreated control cells are represented by the black-filled histogram. Cells pretreated with Tamiflu and stimulated with NGF are depicted by the gray-filled histogram. Cells treated with NGF are depicted by the unfilled histogram. The mean fluorescence for each histogram is indicated for 98% gated cells (see Figure 5A).

Western blot

TrkA-PC12 cells were grown in 75-cm² flasks at 90% confluence. Cells were treated with 500 μM Tamiflu in serum free medium for 24 h or left untreated. Cells were stimulated with 50 ng/mL NGF for 15 min and immediately lysed in 500 μL of 1 × SDS sample buffer. To each cell lysate was added 3%
2-mercaptoethanol and boiled for 10 min. Cell lysates were resolved by 8% SDS-PAGE gel, and the blots probed with polyclonal goat anti-pY490 Trk antibody followed by HRP conjugated secondary anti-goat IgG antibody, and Western Lightning Chemiluminescence Reagent Plus. Sample concentration for gel loading was determined by BioRad reagent.

Sialidase activity in viable cells
TrkA-PC12, TrkA-deficient PC12\textsuperscript{nnr5} cells or primary cortical neurons were grown on 12-mm circular glass slides precoated with poly-D-lysine in a medium containing 5% horse and 4% fetal calf sera. After removing the medium, 2.04 mM 4-MUNANA (4-MU) substrate [2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid] (Sigma) in Tris buffered saline pH 7.4 was added to each well alone (Ctrl), with 50 ng/mL NGF for TrkA expressing cells and 100 ng/mL BDNF for primary cortical neurons, or in combination of NGF or BDNF and 200–500 μM Tamiflu (see Figures 7 and 9). Additionally, TrkA-PC12 cells were pretreated with 100 μg/mL MAL-2 for 45 min or left untreated. After removing medium and washing, 2.04 mM 4-MUNANA substrate in Tris buffered saline pH 7.4 was added to each well either alone or in combination with 50 ng/mL NGF or 100 ng/mL BDNF. Additionally, the medium (20 μL) was taken from the cell wells and added to 20 μL of 4-MUNANA substrate. A positive control sialidase (Clostridium perfringens) (Sigma) (10 μL; with a specific activity of 1 U per 1.0 μmol of N-acetylneuraminic acid per minute) was added to 20 μL of 2.04 mM 4-MUNANA substrate. The substrate is hydrolyzed by sialidase to give free 4-methylumbelliferone which has a fluorescence emission at 450 nm (blue color) following an excitation at 365 nm. Fluorescent images were taken after 1–10 min using epi-fluorescent microscopy (40× objective). The data are a representation of one out of five independent experiments showing similar results.

NGF-induced sialidase activity in cell suspensions of TrkA-PC12 cells
TrkA-PC12 cells at 90% confluency in 25-cm\textsuperscript{2} flasks were resuspended in 500 μL of serum-free medium. To 100 μL or 400 μL of cell suspension (3–4 × 10\textsuperscript{6} cells/mL) was added 50 ng/mL NGF at different times and the remaining cells were left untreated as controls. The NGF-treated and untreated cells were added (30 × 10\textsuperscript{6} total cells) to substrate buffer (20 mM Tris and 30 mM NaCl) containing aprotinin, leupeptin, PMSF, and 0.05 mM 4-MUNANA in cuvets with or without the presence of 200 μM of either Tamiflu, BCX-1812, or BCX-1827. The fluorescence intensity readings were immediately taken over 15 min using the Varian Cary Eclipse Fluorescence Spectrophotometer at low and medium PMT voltage detector settings. The substrate, 4-MUNANA, is hydrolyzed by sialidases to give free 4-methylumbelliferone which has a fluorescence emission at 450 nm following an excitation at 365 nm. The sialidase activity in the cell samples was calculated from a standard sialidase activity curve, corrected for background endogenous sialidase activity in untreated cells, and expressed as nano Units per mg of 4-MUNANA for a total of 3 × 10\textsuperscript{7} cells.

Statistics
Comparisons between two groups were made by one-way ANOVA at 95% confidence interval using the Bonferroni multiple comparison test, or the Dunnett multiple comparison test for comparisons among more than two groups.

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Conflict of interest statement
The authors declare that they have no competing financial interests.

Abbreviations
BDNF, brain-derived neurotrophic factor; NGF, nerve growth factor; PC12, rat pheochromocytoma cell line; PC12\textsuperscript{nnr5}, a tyrosine kinase deficient mutant, (NGF nonresponsive); PMSF, phenylmethanesulfonyl fluoride; TBS, tris buffered saline; TrkA, PC12 cells overly expressing human TrkA receptors; TrkB-nnr5 or nnr5-TrkB cell line, PC12nnr5 stably transfected with TrkB receptors; TSA\textsuperscript{Asp98-Glu}, mutant TS with glutamic acid residue at position 98 replacing aspartic acid; TS, trans-sialidase.

References


