Neuroglycan C Is a Novel Midkine Receptor Involved in Process Elongation of Oligodendroglial Precursor-like Cells*

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Midkine is a heparin-binding growth factor that promotes cell attachment and process extension in undifferentiated bipolar CG-4 cells, an oligodendroglial precursor cell line. We found that CG-4 cells expressed a non-proteoglycan form of neuroglycan C, known as a part-time transmembrane proteoglycan. We demonstrated that neuroglycan C before or after chondroitinase ABC treatment bound to a midkine affinity column. Neuroglycan C lacking chondroitin sulfate chains was eluted with 0.5 M NaCl as a major fraction from the column. We confirmed that CG-4 cells expressed two isoforms of neuroglycan C, I, and III, by isolating cDNA. Among three functional domains of the extracellular part of neuroglycan C, the chondroitin sulfate attachment domain and acidic amino acid cluster box domain showed affinity for midkine, but the epidermal growth factor domain did not. Furthermore, cell surface neuroglycan C could be cross-linked with soluble midkine. Process extension on midkine-coated dishes was inhibited by either a monoclonal anti-neuroglycan C antibody C1 or a glutathione S-transferase-neuroglycan C fusion protein. Finally, stable transfectants of B104 neuroblastoma cells overexpressing neuroglycan C-I or neuroglycan C-III attached to the midkine substrate, spread well, and gave rise to cytoskeletal changes. Based on these results, we conclude that neuroglycan C is a novel component of midkine receptors involved in process elongation.

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2 The abbreviations used are: MK, midkine; mMK, mouse MK; PTN, pleiotrophin; CS, chondroitin sulfate; CS-E, chondroitin sulfate E; NGC, neuroglycan C; EGF, epidermal growth factor; PG, proteoglycan; AB, acidic amino acid cluster box; HEK293T, human embryonic kidney 293T; FN, fibronectin; FITC, fluorescence isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; PBS, phosphate-buffered saline; TBS-T, Tris-buffered saline containing Tween 20; CHAPS, 3-[3-cholamidopropyl]dimethylammoniomio]propanesulfonic acid; GST, glutathione S-transferase; HBS, Hepes-buffered saline; mH, Myc-His; HA, hemagglutinin.

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A. NGC-I and NGC-III are alternatively spliced variants with different cytoplasmic domains. For the expression of a full-sized recombinant NGC-I and NGC-III, a Myc-hexahistidine tag (MycH tag) was attached at the carboxyl-terminal end (not shown). N, amino terminus; C, carboxyl terminus; sig, signal sequence; CS, chondroitin sulfate attachment domain; AB, acidic amino acid cluster box; EGF, epidermal growth factor domain; TM, transmembrane region; aa, amino acids. Arrow, unique chondroitin sulfate attachment site identified by Ref. 23. B, recombinant NGC proteins expressed in this study. CS, A, or EGF domain of NGC with a specific tag.

FIGURE 1. Schematic representation of NGC-I, NGC-III, and recombinant NGC proteins. A. NGC-I and NGC-III are alternatively spliced variants with different cytoplasmic domains. For the expression of a full-sized recombinant NGC-I and NGC-III, a Myc-hexahistidine tag (MycH tag) was attached at the carboxyl-terminal end (not shown). N, amino terminus; C, carboxyl terminus; sig, signal sequence; CS, chondroitin sulfate attachment domain; AB, acidic amino acid cluster box; EGF, epidermal growth factor domain; TM, transmembrane region; aa, amino acids. Arrow, unique chondroitin sulfate attachment site identified by Ref. 23. B, recombinant NGC proteins expressed in this study. CS, A, or EGF domain of NGC with a specific tag.

ExPERIMENTAL PROCEDURES

Cells and Cell Culture—CG-4 cells were maintained on cell culture dishes coated with poly-D-lysine at 5 μg/ml in sterile water. The cells were cultured in Dulbecco’s modified Eagle’s medium/N1 basal medium supplemented with 30% B104 rat neuroblastoma-conditioned medium (CG-4 medium) to maintain a bipolar phenotype (27).

Reagents and Chemicals—Recombinant mouse MK was expressed using baculovirus system and purified with a heparin-Sepharose column (28). Human plasma fibronectin (FN) was purified as described (29). Protease-free chondroitinase ABC from Proteus vulgaris (EC 4.2.2.4) was purchased from Seikagaku Corp. (Tokyo, Japan). Rhodamine-phalloidin and bovine serum albumin in PBS. For the detection of NGC, cells were incubated with either monoclonal anti-NGC antibody C5 (30) or polyclonal rabbit anti-NGC antisera (30). For the staining of tubulin, cells were treated as described (31) and incubated with monoclonal anti-β-tubulin antibody (clone2B8-33, Sigma). Cells were also stained by monoclonal anti-Myc tag antibody (clone 9E10, Upstate Biotechnology, Lake Placid, NY). After reacting with secondary antibodies, cells were mounted in 50% glycerol/PBS containing 2.6% 1,4-diazabicyclo[2.2.2]octane and examined with a laser scanning confocal imaging system (Bio-Rad).

SDS-PAGE and Western Blotting—SDS-PAGE was performed according to Laemmli (32) after reduction with 2-mercaptoethanol. Then, proteins were electrotransferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA) according to Towbin et al. (33). The membrane was incubated with 20 μg/ml Tris-HCl, pH 7.4, containing 150 μg/ml NaCl and 0.1% Tween 20 (TBS-T) with 5% nonfat milk for blocking and incubated with a primary antibody in TBS-T, namely monoclonal anti-NGC C5, monoclonal anti-FLAG M2 antibody (Sigma), monoclonal anti-Myc tag antibody, or monoclonal anti-HA tag antibody (Roche Diagnostics). After extensive washing with TBS-T, membranes were incubated with a horseradish peroxidase-labeled secondary antibody diluted with TBS-T followed by ECL detection reagents (Amersham Biosciences) according to the instruction manual. Signals were visualized on Fuji x-ray film.

Isolation of a PG-rich Fraction from Postnatal 10-day Rat Brains and Chondroitinase ABC Treatment—Membrane-bound proteoglycans were prepared from a PBS-insoluble fraction of 10-day-old (P10) Sprague-Dawley rat brains as described previously (30). To remove chondroitin sulfate chains, samples were digested with chondroitinase ABC at 50 milliunits/ml in 100 mM Tris-HCl, pH 7.4, containing 30 mM sodium acetate and 5 mM EDTA (chondroitinase ABC buffer) for 2 h at 37 °C.

Construction of NGC Expression Vectors for Mammalian Cells—Rat NGC cDNA (GenBank™ accession numbers NM_019284 and NM_133652) was amplified by PCR with specific primers for NGC, 5′-GGGATCCGGCGAATTGGCCGGACGCTG-3′, named primer F1, and 5′-TCATCAGTTGCTAGTTATTCTGGAGAA-3′ (R1650) using oligo(dT) primed CG-4 cDNA as a template. PCR products were subcloned into pGEM-T Easy (Promega Corp., Madison, WI). To construct a Myc-His-tagged NGC-I vector and a Myc-His-tagged NGC-III vector, another PCR using cDNA encoding either NGC-I or NGC-III as a template with primers 5′-GGGATCCCTGGGACGCGACGCCCCAACAGCTGATGG-3′ (F958BM) and 5′-ACTAGTGGTCAAGTATCTGGAGACTCGAGAA-3′ (R1650sp) was performed, generating 692- and 773-bp fragments. They were subcloned into pGEM-T Easy and digested with KpnI and SpeI to give rise to cDNA fragments encoding the cytoplasmic domain of NGC-I or NGC-III. Each of the resulting fragment was ligated into pcDNA3.1/Myc-His(+) cleaved with BamHI and XbaI together with a 1100-bp BamHI-KpnI fragment from NGC-I cDNA, a sequence common to NGC-I and NGC-III.

Domain-specific cDNA clones were obtained by the following PCR: for the full-sized extracellular domain tagged with a FLAG epitope (CSAE-FLAG), the primer F1 and 5′-CTACTAC-
TTGTCATCGTCCTTGTAAATCGAAGTGCGGTGATGATGAGTGACCTC-3' (RFL1280); for the chondroitin sulfate attachment domain (CS domain), F1 and 5'-GACTAGTCTGAGTCCCTGCTGAAG-3' (R635); for the signal sequence, F1 and 5'-AGGTA-CCAAAGGCTCCGTGCGGTACAGCC-3' (R120); for the acidic amino acid cluster box domain (AB domain), 5'-GGGATC-TTCAGACACAAAGGCA-3' (F625) and 5'-GGATCCTGAGCTCGTCCCTGG-3' (R1090); for the EGF domain, 5'-GAGGGCTCTTGAGGGACAGGCCACCCACAGTCPG-3' (F9595T) and RFL1280. The BamHI-EcoRI fragment encoding CSAE-FLAG, the BamHI-EcoRV fragment encoding the signal sequence, and the StuI-SpeI fragment encoding A domain cDNA were ligated into pcDNA3.1/Myc-His(+) cleaved with SmaI and NotI and introduced into pGEX 5X-2 (Amersham Biosciences) cleaved by StuI and NotI. The resulting fragment was ligated into pGEM-T Easy vector and digested by StuI and NotI. The resulting fragment encoding the CS domain, BamHI-EcoRV fragment encoding the signal sequence, and StuI-SpeI fragment encoding A domain cDNA were ligated into pcDNA3.1/Myc-His(+) cleaved with BamHI and XbaI.

Expression of Recombinant Mouse MK Tagged with HA (mMK-HA) in Baculovirus System—To generate a transfer vector encoding mouse MK with an HA epitope, PCR was performed using mouse MK cDNA as a template, with primers, 5'-GCCGGATTCATGCAGCACCGAGGCTTCTTC-3' and 5'-ACTAGCATAATCAGGAACATCATAGTCCTTTCCT-3'. The GST-NG fusion protein and GST protein were induced by 0.4 mM isopropyl-1-thio-β-D-galactopyranoside and isolated on a glutathione-Sepharose column, and the eluates were further purified by passing through a DEAE-Sepharose column.

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MK Binding Assay of NGC—MK affinity column chromatography was performed as described (9). Eluted fractions were analyzed by Western blotting using anti-NGC antibody or tag-specific antibodies. The binding of GST-MK fusion protein to MK was evaluated using the BIAcore 3000 system (BIAcore, AB), using BIAevaluation software (BIAcore).

Cross-linking of NGC—HEK293T cells overexpressing NGC were washed with ice-cold HBS(+), which contained 1 mM CaCl_2 and 1 mM MgCl_2 in HBS (10 mM HEPES-NaOH, pH 7.2, containing 150 mM NaCl), three times, and incubated with HBS(+) containing mMK-HA at 1 μg/ml for 2 h at 4 °C. After being washed with 0.1% bovine serum albumin in HBS(+), cells were treated with 0.27 mM disuccinimidyl suberate (Pierce) for 15 min at 4 °C, and 1 mM Tris-HCl, pH 7.4 was added to 20 mM as a final concentration. Then, cells were lysed in lysis buffer, which contained 0.6% CHAPS, 0.1% sodium deoxycholate, 5 μg/ml 4-(2-aminoethyl)-benzenesulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 mM pepstatin A in HBS.

Assay for Process Extension—Each well of a Falcon 1147 plate (BD Biosciences) was incubated with a coating solution for 2 h at 27 °C and washed with sterile water three times. CG-4 cells were trypsinized, suspended with 0.1% soybean trypsin inhibitor in PBS, washed with PBS two times, and seeded at 4 × 10^4 cells/well in CG-4 medium. CG-4 cells were incubated with a reagent for 10 min and seeded on the MK-coated wells. After 30 min, photographs of five different areas in each well were taken. Based on the photographs, the percentage of cells with processes was quantified. NGC-overexpressing B104 cells and mock-transfected cells were incubated in Dulbecco’s modified Eagle’s medium overnight, trypsinized, suspended with 0.1% soybean trypsin inhibitor in PBS, washed with PBS two times, seeded on the wells of Falcon 1147 plates coated with mMK, and also analyzed for process extension.

RESULTS

CG-4 Cells Express NGC-I and NGC-III as a Non-PG Form—First, we examined the expression of NGC in bipolar CG-4 cells by immunofluorescence microscopy using monoclonal anti-NGC antibody C5 (Fig. 2A). NGC was located in the cell processes and the basal parts of these processes. Then, to study whether NGC expressed in CG-4 cells carries chondroitin sulfate chains, NGC was immunoprecipitated from lysate of CG-4
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cells with polyclonal anti-NGC antibodies, incubated with chondroitinase ABC or with buffer alone, and analyzed by Western blotting (Fig. 2B). NGC from CG-4 cells behaved as a 100-kDa band on the SDS-PAGE gel irrespective of chondroitinase ABC treatment, whereas NGC from P10 rat brain was 180 kDa before chondroitinase treatment and 100 kDa after the treatment. These results indicate that CG-4 cells expressed NGC and that the non-PG form was predominant.

Four isoforms due to alternative splicing were reported in NGC (25). To know what isoforms were expressed in CG-4 cells, we performed reverse transcription-PCR using primers specific for the rat NGC sequence and single-stranded cDNA prepared from CG-4 cells as a template and isolated cDNA encoding the open reading frame of NGC. PCR products were subcloned into pGEM-T Easy, and the DNA sequences of 12 randomly chosen clones were analyzed. Nine of the clones were NGC-I, and the others were NGC-III. They all had the same amino acid sequence in the ectodomain but a different 27-amino acid insert in the cytoplasmic part as already reported. We could not detect the PCR product of NGC-II using primers specific for NGC-II.

Binding of Various Forms of NGC to MK—To evaluate the binding of NGC to MK, we performed MK affinity column chromatography using both native NGC and a recombinant NGC lacking glycosaminoglycan chains. First, the lystate of CG-4 cells was applied to an MK-Sepharose column and eluted at 0.2, 0.3, 0.4, 0.5, and 1 m NaCl. SDS-PAGE and subsequent Western blotting revealed that NGC was eluted with 0.5 m NaCl (Fig. 3A).

Second, the PG-rich fraction from P10 rat brain was applied to an MK-Sepharose column before or after chondroitinase ABC treatment (Fig. 3B). Intact NGC was mainly eluted with 0.5 and 0.6 m NaCl, but minor fractions eluted with 0.7 and 1.0 m NaCl were present. After chondroitinase ABC treatment, NGC was mainly eluted with 0.5 m NaCl. These results showed that native NGC from P10 rat brain bound to MK more strongly than that lacking glycosaminoglycan chains. Third, to establish that NGC without glycosaminoglycan chains can bind to MK, we expressed GST-NGC and GST proteins in bacteria and purified them (Fig. 3C). The binding affinity of GST-NGC and GST proteins was analyzed by surface plasmon resonance spectroscopy (Fig. 3D). We confirmed that GST-NGC bound to MK, but GST did not. BIAevaluation software gave the $K_d$ of GST-NGC to MK as 51.8 nM from the values of association and dissociation. The result indicates that MK and NGC could bind with protein-protein interaction.

CS and AB Domains of NGC Can Bind to MK—The extracellular region of both NGC-I and NGC-III is composed of three functional domains: a chondroitin sulfate attachment domain, an AB domain, and an EGF domain. To further narrow down the MK-binding site in NGC, we expressed recombinant NGC proteins containing a specific domain as secreted forms with a tag (Fig. 1B). Each expression vector was transfected into B104 rat neuroblastoma cells. Culture medium containing transiently expressed recombinant NGC proteins was harvested, applied to an MK-Sepharose column, and eluted with increasingly higher concentrations of NaCl. Bound fractions were analyzed by Western blotting using specific anti-tag antibodies (Fig. 4). We confirmed that all of the four recombinant NGC proteins were successfully expressed and secreted by the cells. Dosed sizes of the core protein of CSAE-FLAG, CS-MycH, and A-MycH were 40, 17, and 10 kDa, respectively, whereas they were 90, 120, and 35 kDa on the gel. Recombinant NGCs may have some modifications such as N-glycosylation and O-glycosylation, as found in NGC from P10 rat brain (26). CSAE-FLAG, CS-MycH, and A-MycH were capable of binding to the MK column and were eluted from it with 0.3 and 0.5 m NaCl, whereas E-FLAG did not bind. These results show that MK could bind to two different domains of NGC. It should be noted that the NaCl concentration required for the elution of these mutants was lower than that required for the elution of intact NGC.
Binding of NGC-I and NGC-III to MK—It is necessary to demonstrate that cell surface NGC binds to MK if NGC serves as an MK receptor. For this purpose, we performed cross-linking experiments to examine the binding of cell surface NGC to the soluble form of MK. Since CG-4 cells did not express enough NGC to do the cross-linking, we overexpressed NGC-I or NGC-III tagged with Myc-His (I-mH and III-mH, respectively) in HEK293T cells (Fig. 5A). NGC-III was slightly larger than NGC-I as NGC-III had a 27-amino acid insertion relative to NGC-I. After chondroitinase ABC treatment, neither NGC-I nor NGC-III changed size on the gel (Fig. 5A, arrowhead), indicating that chondroitin sulfate chains were not attached to NGCs in HEK293T cells. The broad bands may be caused by heterogeneity in N- and O-glycosylation. Furthermore, to detect the binding of MK more clearly, recombinant mouse MK with an HA tag was expressed by the baculovirus system (Fig. 5B). Mouse MK-HA was added to the culture of HEK293T cells overexpressing NGC-I or NGC-III or to that of mock-transfected cells and cross-linked by disuccinimidyl suberate (DSS). Cell lysates were immunoprecipitated using anti-NGC antibodies and subjected to Western blotting (Fig. 5C). NGC-I and its cross-linked complex were detected as 110-, 130-, and 250-kDa bands by the anti-Myc antibody, whereas NGC-I, which formed a complex with MK-HA, was detected as 130- and 250-kDa bands by the anti-HA antibody (Fig. 5C). These bands were not detected in controls, namely on mock transfection, elimination of the cross-linking reagent, and elimination of the addition of MK. The bands of 100 and ~66 kDa appeared also in controls and were nonspecific. The cross-linked 130-kDa band was broad, partly due to the broad nature of neuroglycan C before cross-linking and probably also due to the fact that in addition to MK monomer, its dimer is present in significant amounts (34) and may be involved in binding to NGC. Similar results were obtained from cross-linking of MK-HA to NGC-III (data not shown). Considering that the 120-kDa band represents a mature form of NGC, we concluded that cell surface NGC-I and NGC-III bind to soluble MK by protein-protein interaction. It is possible that the 250-kDa band represents a dimeric form of the NGC-MK complex.

NGC Is Involved in Process Elongation in CG-4 Cells on MK—CG-4 cells did not attach to uncoated dishes but attached and extended processes on MK-coated dishes. We studied the physiological role of the NGC-MK interaction in the cells cultured on the MK substrate by conducting an inhibition assay with GST-NGC protein since GST-NGC was demonstrated to bind to MK as described above. CG-4 cells were suspended with GST-NGC or GST and seeded on MK-coated dishes. Almost all
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FIGURE 6. GST-NGC fusion protein and monoclonal anti-NGC antibody C1 prevent process extension in CG-4 cells seeded on MK-coated wells. CG-4 cells were seeded on MK-coated wells and photographed as described under “Experimental Procedures.” A, CG-4 cells were incubated with either GST-NGC or GST proteins at 600 nM and incubated for 30 min. B, GST-NGC protein blocked process extension in a dose-dependent manner. The percentage of cells with processes was quantified and expressed as the mean ± S.D. for three independent experiments. C, cross-linking of NGC with mouse MK-HA. Cells were treated with monoclonal antibody C1 (100 μg/ml) or mouse IgG (100 μg/ml) and seeded on MK as a substrate. D, process extension was inhibited by C1 antibody. The percentage of cells with processes elongating longer than 10 μm from the cell body was quantified and expressed as the mean ± S.D. for three independent experiments.

cells attached to the substrate with or without GST-NGC. However, the subsequent extension of cell processes was blocked by GST-NGC in a dose-dependent manner but not by GST (Fig. 6, A and B). With the addition of 600 nM (50 μg/ml) GST-NGC, only 24% of CG-4 cells extended processes as compared with 95% at the same concentration of GST and 98% in the wells with no reagent. Furthermore, the monoclonal anti-NGC antibody C1, recognizing the amino-terminal CS domain (30), strongly inhibited the extension, but control mouse IgG did not (Fig. 6, C and D). These results indicated that cell surface NGC was involved in process extension rather than cell adhesion in CG-4 cells, and the amino-terminal CS domain and AB domain seem to be important for this function, in agreement with the results of MK affinity column chromatography.

NGC-overexpressing Cells Preferentially Spread on MK—To further study the functional significance of interactions between NGC and MK, stable transfectants of NGC-I-MycH and NGC-III-MycH were raised in B104 neuroblastoma cells (designated as I-mH and III-mH). The expression of NGC was evaluated by Western blotting and immunofluorescent staining using specific anti-tag antibodies. As in the HEK293T cells, both I-mH and III-mH were detected as bands of 100 and 120 kDa, lacking chondroitin sulfate chains (data not shown). I-mH and III-mH were each mainly immunodetected at the cell surface as expected (Fig. 7A). Interestingly, I-mH cells or III-mH cells appeared to have a tendency to spontaneously extend processes even without serum deprivation. When these NGC-overexpressing cells were seeded in the wells coated with either FN or mouse MK, almost all cells attached to the MK substrate within 15 min, whereas only a few cells attached to the FN substrate. I-mH cells and III-mH cells spread on MK became flattened by 30 min, and mock-transfected cells attached but did not spread (Fig. 7B). There were still a number of cells floating in the wells coated with FN. Most cells attached to the FN substrate later than 1 h. The ratio of flattened cells to attached cells was 72% in III-mH-overexpressing cells, 33% in I-mH-overexpressing cells, and 9% in mock-transfected cells (Fig. 7C). These results indicate that cell spreading was promoted by NGC-I and NGC-III on the MK substrate. By 1 h, III-mH cells had further changed shape, exhibiting filopodia-like processes (Fig. 7D). I-mH cells slowly became flattened and formed short arms. Filamentous actin seemed to be accumulated at the tips of these arms. On the other hand, mock transfectants did not change shape except for the flattening caused by spreading.

DISCUSSION

Our results provide convincing evidence that NGC acts as an MK receptor in a specific function, the elongation of cell processes. We demonstrated the binding between soluble MK and cell surface NGC and also between the recombinant ectodomain of NGC expressed as a soluble form and solidified MK. We found that NGC protein without chondroitin sulfate chains could bind to MK relatively strongly as shown by its elution from the MK column with 0.5 M NaCl. The $K_d$ of the binding between MK and GST-NGC fusion protein expressed in bacteria was estimated to be 51.8 nM by surface plasmon resonance spectroscopy. This value is comparable with that between MK and CS-E, namely 46 nM (35). We also found that NGC bound to MK via two domains in the extracellular region, the CS domain and AB domain. Furthermore, stable transfectants of B104 cells overexpressing NGC-I or NGC-III spread faster than mock-transfected cells. Notably, NGC-III-overexpressing cells formed filopodia, suggesting that NGC is involved in MK signal transduction. The binding between MK and NGC was stronger when
chondroitin sulfate chains were attached to NGC. This is probably due to the presence of CS-E units, which bind strongly to MK (35, 36). The chondroitin sulfate portion of NGC is known to have a higher level of the E-unit than neurocan or phosphacan (26). In vivo, it is highly likely that chondroitin sulfate chains, especially those with E-units, enhance signal reception.

MK promotes cell attachment and process elongation in embryonic neural cells (5–7). We found that neither anti-NGC antibody nor GST-NGC fusion protein inhibited the attachment of the cells, but both inhibited the elongation of the processes. The specific role of NGC in process elongation is consistent with the restricted distribution of NGC in nerve tissue. In CG-4 cells, a proteoglycan such as protein-tyrosine phosphatase is involved in the attachment of cells to MK-coated dishes as the binding is strongly inhibited by the addition of heparin or CS-E (19). The MK receptor has multiple components and is likely to form a molecular complex (18). The present result suggests that to realize a cell-specific effect such as process elongation, a receptor with cell-specific expression is utilized in cooperation with other components of the MK receptor.

NGC has been shown to be processed, yielding a truncated form with an exposed EGF domain, and neuronal depolarization facilitates the processing (37). It is known that the EGF domain of NGC activates ErbB3 as a direct ligand (38). Furthermore, a deficiency of NGC causes functional abnormalities of synapses in the early postnatal period (37). Therefore, NGC is an important modulator of neural function at least at certain developmental stages. It is tempting to speculate that NGC is in the receptor complex for MK and modulates the role of the complex to realize a specific function, process elongation.

The function of NGC is expected to differ depending upon the type of the variant expressed in the cell. In this respect, it should be mentioned that NGC-I was the major variant expressed in CG-4 cells as it is in the rat brain (22, 39).

MK is broadly expressed in the nervous system at embryonic stages; even after birth, the developing cerebellum expresses it (1, 2, 40). NGC may be involved in the elongation of processes not only in oligodendrocyte precursor cells but

![Image of Figure 7](https://example.com/image)
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also in other cells in the nervous system. It should be kept in mind that MK has about 50% sequence identity with PTN (3,4). CG-4 cells attach and extend processes on PTN as well as on MK (19). Thus, the possible role of NGC in the actions of PTN in the nervous system is also worth investigating.

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