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Neuroglycan C, a brain-specific part-time proteoglycan, with a particular multidomain structure

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Neuroglycan C (NGC) is a transmembrane-type of chondroitin sulfate proteoglycan that is exclusively expressed in the central nervous system. NGC gene expression is developmentally regulated, and is altered by addiction to psychostimulants and by nerve lesion. Its core protein has a particular multidomain structure differing from those of other known proteoglycans, and this protein is modified post-translationally in various ways such as phosphorylation and glycosylation. NGC is a novel part-time proteoglycan that changes its structure from a proteoglycan form to a non-proteoglycan form without chondroitin sulfate chains during the development of the cerebellum and retina. Results obtained from immunohistological, cell biological and biochemical experiments suggest that NGC is involved in neuronal circuit formation in the central nervous system. To verify the proposed functions of NGC in the brain, production and phenotype-analyses are being performed in mice with various NGC gene mutations causing the expression or glycosylation of NGC to be altered. ***Published in 2004.***

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Introduction

Proteoglycans (PG) are a group of glycoproteins bearing sulfated glycosaminoglycan (GAG) chains. In the central nervous system (CNS), there are many PG species, some of which are classified into families with a domain structure common to their core proteins [1]. Evidence has accumulated to implicate nervous tissue PGs in the development and injury repair of CNS [1–3]. Of these nervous tissue PGs, neuroglycan C (NGC) is the only membrane-spanning chondroitin sulfate proteoglycan (CSPG) that exists mainly in association with the neuronal structure such as neuropils and dendrites in the CNS [1,4]. A chicken homolog of NGC was identified in the nervous system, and was named chicken acidic leucine-rich EGF-like domain containing brain protein (CALEB) [5]. Interestingly, most NGC molecules in the mature cerebellum and retina, but not in their immature counterparts, exist in a non-PG form without chondroitin sulfate (CS), indicating that NGC is a part-time PG [6,7]. The domain structure of NGC/CALEB is quite different from those of other transmembrane PGs [4,5].

Transmembrane glycoproteins are generally considered to be involved in outside-to-inside and inside-to-outside signaling of cells. In addition, ectodomain shedding sometimes occurs on the cell surface by proteolysis, and the released ectodomain exerts an influence on other cells changing their physiological states. The best-known example of ectodomain shedding is the epidermal growth factor (EGF) family molecules [8]. Thus, a bifunctional role is proposed for some membrane-spanning glycoproteins as a receptor and a ligand. Since NGC has a multidomain structure distinct from other neuronal transmembrane glycoproteins, it would be expected to participate in certain cellular events in neurons in a particular way.

To elucidate the involvement of NGC in the development and injury repair of the CNS, the structure, expression pattern, molecular interactions, and effects on neuronal cell behaviors have been investigated extensively. In this review, we summarize results obtained from these studies, and present some unpublished results related to the biological meaning that NGC is a part-time PG.

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NGC gene and splice variants

The NGC gene of the mouse (*Cspg5*) comprises six exons, and is approximately 17 kilobases in size [6]. It was assigned to the mouse chromosomal band 9F1 by fluorescence *in situ* hybridization, and the human NGC gene was mapped to the chromosomal band 3p21.3 [9]. Cloning studies with mouse NGC cDNA demonstrated the expression of three distinct splice variants (NGC-I, -II, and -III), two of which (NGC-I and -III) have been identified as CSPGs in the brain. NGC-I is the major variant (over 90% of total), and the homologies of its amino acid sequence are 94.3% and 85.5% with the rat and human counterparts, respectively [4,6,9].

The entire core protein of mouse NGC-I consists of 509 amino acid residues plus a 30-residue signal peptide [6], and is divided into five structurally different domains; an N-terminal domain to which a chondroitin sulfate chain is attached, an acidic amino acid cluster, a cysteine-rich domain containing a single EGF module, a membrane-spanning segment, and a C-terminal cytoplasmic domain of 95 amino acids (Figure 1). NGC-I and -III have a common ectodomain, but NGC-III has a short peptide insert encoded by exon 5 in the middle part of the cytoplasmic domain of NGC-I. The short insert is composed of 27 amino acid residues, and its sequence is well conserved among the mouse, rat and human.

Phosphorylation and glycosylation

The NGC core protein is modified post-translationally in various ways such as phosphorylation, *O*- and *N*-glycosylation, and CS glycosylation. We found that serine residue(s) in the NGC ectodomain could be phosphorylated both intracellularly and extracellularly, and that the kinase activity has a specificity similar to that of casein kinase II [10].

Peptide motif analysis using a computer algorithm demonstrated a cluster of potential *O*-glycosylation sites and three potential *N*-glycosylation sites on the NGC ectodomain (Figure 1). In fact, the NGC core glycoprotein is recognized by various lectins which recognize a particular carbohydrate structure found in *O*- or *N*-glycans [11]. Lectins reactive with NGC do not always recognize other brain-specific CSPGs, neurocan and phosphacan, and *vice versa*, even though they were isolated from the brain at the same developmental stage. The NGC core glycoprotein also has an HNK-1 carbohydrate epitope on its *N*-glycans. It is of interest that the reactivity of NGC with lectins and with an HNK-1 antibody markedly changes as the brain matures [11].

In the ectodomain of the NGC core protein, there are six potential CS glycosylation sites conserved among the mouse, rat and human. We identified the CS-attachment site as Ser-123 in the mouse NGC by site-directed mutagenesis. The CS disaccharide composition of NGC purified from 10-day-old rat brains is significantly different from those of neurocan and phosphacan, *i.e.* higher levels of 4-sulfate unit (about 80% of total) and E unit (about 4%), a highly sulfated disaccharide unit, although neurocan and phosphacan show an almost identical disaccharide composition [11]. The levels of both 6-sulfate and E units of NGC decreased with a compensatory increase in 4-sulfate unit with development of the rat brain. A CS preparation from squid cartilage is abundant in E units (about 60% of total disaccharide units), and this commercial CS preparation (CS-E) exhibits neurite outgrowth promoting activities toward fetal rat hippocampal neurons [12], and interacts with various heparin-bainding growth factors [13]. Although the content of E units in the CS disaccharide units of NGC is much less than that of commercial CS-E, it is likely that NGC participates in signaling of some heparin-binding growth factors through the interactions of its CS chain with these growth factors.

NGC exists in a PG form with a single CS chain in various regions of the developing CNS, while most NGC molecules exist in a non-PG form in the mature cerebellum and retina [6,7]. In other regions of the mature CNS, most NGC molecules exist in a PG form. This finding suggests that the CS glycosylation of NGC is a part of the molecular cascade governing cerebellar and retinal development.

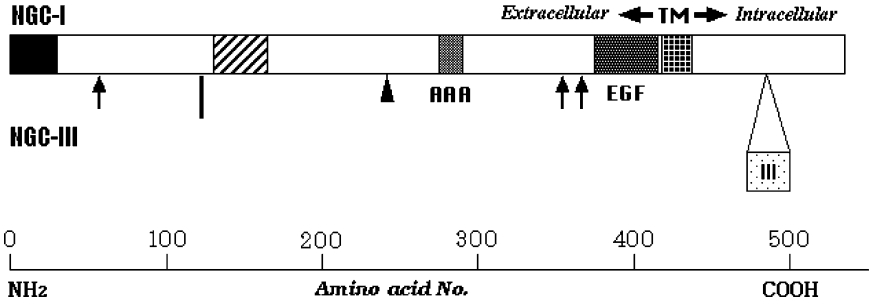

Figure 1. Schematic representation of the structure of two splice variants of mouse NGC core protein. NGC-I is the major variant accounting for more than 90% of total NGC in the developing brain, and NGC-III has a short peptide insert (III) composed of 27 amino acid residues encoded by exon 5 in the cytoplasmic domain. The putative signal sequence is shown as a black box, and a cluster of potential *O*-glycosylation sites exist in the diagonally striped box. A solid vertical bar, a CS-attachment site; arrows, potential *N*-glycosylation sites; an arrowhead, a putative phosphorylation site of serine; AAA, an acidic amino acid cluster; EGF, an epidermal growth factor-like domain; TM, a transmembrane domain.

To investigate the CS glycosylation process of NGC, a cell line expressing a PG form of NGC would be a favorable experimental model. To establish such a cell line, we transfected an NGC cDNA into several cell lines. Unexpectedly, only Neuro 2a cells, a mouse neuroblastoma-derived cell line, expressed NGC bearing CS chains. Other cells such as COS-1 and PC12D cells expressed only a non-PG form of NGC, although they synthesized other CSPGs. These preliminary results suggest that the CS glycosylation of NGC is cell-type dependent and regulated in a manner different from that to other CSPGs.

Tissue-specific expression and localization

Expression of NGC mRNA is restricted to the CNS, and the signal for NGC mRNA in the developing brain is more intense than that in the mature brain [4], suggesting that NGC is involved in cerebral development. Immunohistochemical studies using an antibody against the NGC ectodomain that recognizes both the NGC variants, NGC-I and -III, showed that immunostains were largely observed in association with neuronal structures such as neuropils, dendrites and some nerve fibers mostly in a punctate fashion rather than glial structures in the developing CNS [5–7]. However, this does not rule out the possibility that glial cells express NGC *in vivo*. Our preliminary experiment demonstrated NGC expression by primary cultured astrocytes, suggesting that glial cells in the brain can synthesize NGC at least in some pathological states.

The cerebellum is composed of several types of neurons. Of these cells, only Purkinje cells are stained with the anti-NGC antibody [6]. Purkinje cells form synapses with two major fiber systems; the climbing fiber system and the parallel fiber system. At almost the same stage of cerebellar development, the climbing fibers selectively adhere to and form synapses with the large stems of the Purkinje cell dendrites, whereas the parallel fibers exclusively form synapses with spiny branchlets of Purkinje cell dendrites. Developmental change in the localization of NGC on the Purkinje cells correlates well with synaptogenesis of the climbing fiber system with Purkinje cells. These findings suggest that NGC expression on Purkinje cells mediates adhesion and/or synaptogenesis of the climbing fibers with the Purkinje cells.

Immunostaining of cultured neuronal cells with the anti-NGC antibody demonstrated that NGC was concentrated in dendritic filopodia [7], which are considered to develop into neurites or postsynaptic spines. Therefore, it is again conceivable that NGC is implicated in neuritogenesis and/or synaptogenesis.

Expression changes elicited by drug-addiction and nerve injury

Expression of NGC in the rat CNS increases rapidly in late pregnancy, reaches a peak around the third postnatal week, then decreases to about half of the peak level at adulthood [4,7]. This indicates that NGC expression is developmentally regulated in the CNS. In addition to the developmental change, NGC expression also changes under certain pathological states. A study including DNA macroarray technology demonstrated that up-regulation in NGC protein and mRNA occurred in the corticolimbic circuitry after withdrawal from chronic cocaine treatment [14]. Since addiction to psychostimulants elicits behavioral change in animals, this up-regulation in NGC would contribute to the drug-induced behavioral change. In other words, NGC may be involved in maintenance of brain functions.

NGC expression is also regulated dynamically after optic nerve lesion [15]. NGC/CALEB mRNA is expressed in most developing retinal ganglion cells (RGC), and by 30–50% of all RGCs in the mature retina. After optic nerve lesion in the adult rat, the number of NGC/CALEB mRNA-positive RGCs decreases very rapidly to values below 10% of the total number of surviving RGCs, but the number again increases 1 week after injury to over 60% of the surviving cells. Under conditions for nerve fiber regeneration, about one-third of the regenerating RGCs express NGC/CALEB mRNA. These findings suggest that NGC participates in the repair processes of injured neuronal circuits of CNS, at least of injured nerve fibers. It should be examined whether NGC protein exists on regenerating nerve fibers, and if so, which molecular form of NGC exists, a PG form or a non-PG form.

Involvement in neuritogenesis

As discussed above, some circumstantial evidence of NGC involvement in neuritogenesis has been accumulated. To study this more directly, neurite outgrowth assays were performed in a permissive environment using embryonic chicken tectal cells in the presence or absence of Fab fragments of anti-NGC/CALEB antibodies [5]. As expected, neurite length was dramatically diminished depending on the Fab concentration applied. Recently, we found that the addition of a recombinant NGC ectodomain expressed in *E. coli* to primary cultures of fetal rat neocortical neurons stimulated significant neurite outgrowth from these cells. Both experiments gave further circumstantial, but somewhat more direct evidence to support involvement of NGC in neuritogenesis. However, neither interactive partners of NGC at dendritic filopodia and growth cones nor signaling pathways downstream of NGC that modulate neurite elongation have yet been identified.

Molecules that interact with NGC

Search for molecules that bind to NGC is essential to elucidate both functions and intracellular transport of NGC. It has been reported that NGC/CALEB binds to two adhesion-regulating extracellular matrix molecules, tenascin-C and tenascin-R, via its acidic domain [5,15]. Tenascins modulate neurite outgrowth and formation of actin-rich microprocesses along neurite shafts, or dendritic filopodia [16]. This, together with the fact that NGC

is concentrated at dendritic filopodia [7], suggests that the interaction of NGC with tenascins at the surface of dendritic filopodia is implicated in neuritogenesis. Besides interaction with tenascins, our preliminary studies demonstrated that a recombinant EGF module of NGC bound to and activated a subtype of EGF receptor tyrosine kinases [17], suggesting that NGC is a new member of the EGF/neuregulin family. These molecular interactions of the NGC ectodomain could be modified by its post-translational modifications described above, but the manner of the modification remains to be examined.

The juxtamembrane cytoplasmic short peptide domain of NGC/CALEB has been shown to interact with the Golgi-associated protein, PIST (PDZ domain protein interacting specifically with TC10), an interaction partner of the small GTPase TC10 [18]. Since PIST is important for the transport of some membrane proteins from Golgi bodies to the plasma membrane, NGC may be transported with PIST to the cell surface. It would be of interest to determine the molecular interactions that mediate the transport of NGC to and the concentration of NGC in dendritic filopodia.

Future perspectives

Immunohistochemical and cell biological studies have suggested the involvement of NGC, a transmembrane CSPG, in some steps of neural network formation in CNS such as neurite elongation and synaptogenesis as discussed above. NGC should regulate these cellular processes at dendritic filopodia and growth cones through interactions with both cell surface microenvironment and cytoskeletal system. Tenascins and PIST have been identified as interactive partners of the ectodomain and the cytoplasmic domain of NGC, respectively [5,18]. However, it is obviously impossible to fully understand the function of NGC only by investigating these molecular interactions. Further search for NGC-binding molecules should be performed in the near future. Since NGC-I and -III differ in the structure of the cytoplasmic domain and in the destination of the intracellular transport; dendrites for NGC-I and axons for NGC-III [19], identification of binding molecules specific to each cytoplasmic domain would also take an important step towards elucidating the mechanism for differential intracellular transport in neurons. Yeast two-hybrid system and phage display system must be useful techniques for this purpose.

NGC is modified by *O*- and *N*-glycosylations as well as by CS glycosylation as described above. These glycan moieties would regulate functions of the NGC core protein through interactions with or modification of a functional peptide domain of NGC or through interactions with an NGC-binding molecule. In addition, it is considered that some glycans themselves function independently of the core protein. Identification of molecules whose affinities to NGC vary depending on the glycan moieties of NGC is important and urgent. Phage display may be a favorable method, because this method can be applicable to search for molecules that bind to carbohydrates as well as proteins.

Molecules expressed restrictedly in CNS are generally presumed to be implicated in the maintenance of brain structure and function, so that aberrant expression of these molecules would result in abnormal brain structure and function leading to altered behaviors, loss of memory, loss of learning capability and so on. We are currently attempting to produce some NGC gene mutant mice to elucidate the functions of NGC *in vivo*. A mouse strain with a low expression of NGC (about 6% of the control) produced to date showed low motility under a certain genetic background. This, together with the results observed in drug-addiction [14], suggests that NGC is implicated in the maintenance of brain functions. This also suggests the existence of human genetic diseases with aberrant NGC expression, although there have not been any genetic neuronal or psychiatric disorders assigned to 3p21.3, the chromosomal site of the NGC gene (*CSPG5*). Furthermore, to clarify the biological significance of NGC being a part-time PG, production of a mouse strain expressing only the non-PG form of NGC, but not the PG-form of NGC, would be useful.

Expression pattern of NGC/CALEB mRNA in the retina after optic nerve lesion is suggestive of the application of NGC to the regeneration of optic nerve fibers. Our preliminary results that an exogenously added NGC ectodomain promotes neurite elongation from cultured neocortical neurons also suggest a potential application of the active domain of NGC to CNS regeneration, which is considered impossible at present.

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References

1 Bandtlow CE, Zimmermann DR, Proteoglycans in the developing brain: New conceptual insight for old protein, *Physiol Rev* **80**, 1267–90 (2000).
2 Oohira A, Matsui F, Tokita Y, Yamauchi S, Aono S, Molecular interactions of neural chondroitin sulfate proteoglycans in the brain development, *Arch Biochem Biophys* **374**, 24–34 (2000).
3 Morgenstern DA, Asher RA, Fawcett JW, Chondroitin sulphate proteoglycans in the CNS injury response, *Prog Brain Res* **137**, 313–32 (2002).
4 Watanabe E, Maeda N, Matsui F, Kushima Y, Noda M, Oohira A, Neuroglycan C, a novel membrane-spanning chondroitin sulfate proteoglycan that is restricted to the brain, *J Biol Chem* **270**, 26876–82 (1995).
5 Schumacher S, Volkmer H, Buck F, Otto A, Tarnok A, Roth S, Rathjen FG, Chicken acidic leucine-rich EGF-like domain containing brain protein (CALEB), a neural member of the EGF family

of differentiation factors, is implicated in neurite formation, *J Cell Biol* **136**, 895–906 (1997).
6 Aono S, Keino H, Ono T, Yasuda Y, Tokita Y, Matsui F, Taniguchi M, Sonta S, Oohira A, Genomic organization and expression pattern of mouse neuroglycan C in the cerebellar development, *J Biol Chem* **275**, 337–42 (2000).
7 Inatani M, Tanihara H, Oohira A, Otori Y, Nishida A, Honjo M, Kida N, Honda Y, Neuroglycan C, a neural tissue-specific transmembrane chondroitin sulfate proteoglycan, in retinal neural network formation, *Invest Ophthalmol Vis Sci* **41**, 4338–46 (2000).
8 Fowlkes JL, Winkler MK, Exploring the interface between metallo-proteinase activity and growth factor and cytokine bioavailability, *Cytokine Growth Factor Rev* **13**, 277–87 (2002).
9 Yasuda Y, Tokita Y, Aono S, Matsui F, Ono T, Sonta S, Watanabe E, Nakanishi Y, Oohira A, Cloning and chromosomal mapping of the human gene of neuroglycan C (NGC), a neural transmembrane chondroitin sulfate proteoglycan with an EGF module, *Neurosci Res* **32**, 312–22 (1998).
10 Yamauchi S, Tokita Y, Aono S, Matsui F, Shuo T, Ito H, Kato K, Kasahara K, Oohira A, Phosphorylation of neuroglycan C, a brain-specific transmembrane chondroitin sulfate proteoglycan, and its localization in the lipid rafts, *J Biol Chem* **277**, 20583–90 (2002).
11 Shuo T, Aono S, Matsui F, Tokita Y, Maeda H, Shimada K, Oohira A, Developmental changes in the biochemical and immunological characters of the carbohydrate moiety of neuroglycan C, a brain-specific chondroitin sulfate proteoglycan, *Glycoconj J* **20**, 267–78 (2004).
12 Clement AM, Sugahara K, Faissner A, Chondroitin sulfate E promotes neurite outgrowth of rat embryonic day 18 hippocampal neurons, *Neurosci Lett* **269**, 125–8 (1999).
13 Deepa SS, Umehara Y, Higashiyama S, Itoh N, Sugahara K, Specific molecular interactions of oversulfated chondroitin sulfate E with various heparin-binding growth factors. Implications as physiological binding partner in the brain and other tissues, *J Biol Chem* **277**, 43707–16 (2002).
14 Toda S, McGinty JF, Kalivas PW, Repeated cocain administration alters the expression of genes in corticolimbic circuitry after a 3-week withdrawal: A DNA macroarray study, *J Neurochem* **82**, 1290–9 (2002).
15 Schumacher S, Jung M, Norenberg U, Dorner A, Chiquet-Ehrismann R, Stuermer CAO, Rathjen FG, CALEB binds via its acidic stretch to the fibrinogen-like domain of tenascin-C or tenescin-R and its expression is dynamically regulated after optic nerve lesion, *J Biol Chem* **276**, 7337–45 (2001).
16 Zacharias U, Leuschner R, Norenberg U, Rathjen FG, Tanascin-R induces actin-rich microprocesses and branches along neurite shafts, *Mol Cell Neurosci* **21**, 626–33 (2002).
17 Kinugasa Y, Ishiguro H, Ohmoto H, Tokita Y, Oohira A, Higashiyama S, Neuroglycan C, is a ligand of ErbB3 and a new member of the neuregulin family, *Seikagaku* **75**, 1135 (abstract) (2003).
18 Hassel B, Schreff M, Stube E, Blaich U, Schumacher S, CALEB/NGC interacts with the Golgi-associated protein PIST, *J Biol Chem* **278**, 40136–43 (2003).
19 Tokita Y, Aono S, Ida M, Shuo T, Matsui F, Oohira A, Regulation of polarized targeting of a brain-specific chondroitin sulfate proteoglycan, neuroglycan C, in a cultured neuron, 34th *Meeting American Soc Neurosci,* Program No. 742.10 (abstract), (2003).

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