Genomic Organization and Expression Pattern of Mouse Neuroglycan C in the Cerebellar Development*

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Neuroglycan C (NGC) is a membrane-spanning chondroitin sulfate proteoglycan with an epidermal growth factor module that is expressed predominantly in the brain. Cloning studies with mouse NGC cDNA revealed the expression of three distinct isoforms (NGC-I, -II, and -III) in the brain and revealed that the major isoform showed 94.3% homology with the rat counterpart. The NGC gene comprised six exons, was approximately 17 kilobases in size, and was assigned to mouse chromosome band 9F1 by fluorescence in situ hybridization. Western blot analysis demonstrated that, although NGC in the immature cerebellum existed in a proteoglycan form, most NGC in the mature cerebellum did not bear chondroitin sulfate chain(s), indicating that NGC is a typical part-time proteoglycan. Immunohistochemical studies showed that only the Purkinje cells were immunopositive in the cerebellum. In the immature Purkinje cells, NGC, probably the proteoglycan form, was immunolocalized to the soma and thick dendrites on which the climbing fibers formed synapses, not to the thin branches on which the parallel fibers formed synapses. This finding suggests the involvement of NGC in the differential adhesion and synaptogenesis of the climbing and parallel fibers with the Purkinje cell dendrites.

Proteoglycans are a group of proteins that bear covalently bound sulfated glycosaminoglycan chains. They are located on the cell surface and in extracellular spaces in various animal tissues including the central nervous system, and it is now believed that they play pivotal roles in the development, maintenance, and aging of tissues via cell-cell and cell-substratum interactions.

In the vertebrate central nervous system, there are many species of proteoglycans with different structural features (1, 2). This could be due to the existence of a large number of cell types that constitute many neuronal circuits in the brain and to their multiple roles in various cellular events including mitogenesis, migration, differentiation, axonal outgrowth, and synaptogenesis (3–5). Of these neural proteoglycans, some are transmembrane chondroitin sulfate proteoglycans such as NG2 (6) and receptor-type protein tyrosine phosphatase β (7, 8). Both have been reported to exist mainly in the developing mammalian brain and are involved in the signal transduction of growth factors (9–12) as well as cell-substratum interaction.

Recently, we found a new transmembrane chondroitin sulfate proteoglycan, named neuroglycan C (NGC), that is expressed in the brain, especially at the surfaces of neuronal cells, but not in other tissues in rats (13) and humans (14). NGC cDNA cloned from rat brain libraries encodes a membrane protein composed of a signal peptide (30 amino acids) and a core protein of mature NGC (514 amino acids). The core protein is divided into five structurally different domains: an N-terminal domain to which chondroitin sulfate chain(s) may be attached, a cluster of 9 acidic amino acids, a cysteine-containing domain with an EGF-like motif, a membrane-spanning segment, and a C-terminal cytoplasmic domain of 95 amino acids with two potential phosphorylation sites for protein kinase C. Although the precise physiological functions are not known at present, considering that NGC is expressed exclusively in the brain, especially in the immature brain where the neuronal circuits are actively being formed, this proteoglycan may play some roles in neuronal circuit formation.

In this paper, we report that the localization of NGC changes in a developmentally regulated manner in the mouse cerebellum and that the structure of NGC changes from the proteoglycan form to the nonproteoglycan form as the cerebellar development proceeds. Based on these experimental results, we discuss how NGC is involved in the neuronal circuit formation in the cerebellum. Additionally, we describe the isolation of cDNA clones encoding three distinct isoforms of mouse NGC, organization, and chromosomal assignment of the mouse NGC gene.

EXPERIMENTAL PROCEDURES

cDNA Cloning of Mouse NGC—NGC phage clones were isolated from a λ ZAP cDNA library derived from the brain of a 2-month-old C57BL/6 strain mouse using a rat NGC cDNA (13) as a probe. Bacteriophages were plated at 5 × 10⁷ plaque-forming units/95 × 135-mm dish and approximately 5 × 10⁵ plaques were screened. Positive plaques were purified by two additional rounds of screening and then the excision of the plBluescript phagemid from the ZAP vector (Stratagene, La Jolla, CA) was carried out. Both complementary strands of the mouse NGC cDNA were sequenced using BcaBEST Dideoxy Sequencing kit, dCTP version (TaKaRa, Osaka, Japan).

Isolation and Characterization of Mouse NGC Genomic DNA—A mouse NGC cDNA was used as a probe to screen a 129SVJ mouse liver genomic library (Stratagene). Seven positive clones with different XhoI patterns were isolated from approximately 5 × 10⁶ plaques. The clones were mapped by restriction enzyme digestion and Southern hybridization. Restriction fragments that included all exon sequences and intron/exon splice junctions were subcloned into pBluescript phagemid (Stratagene) for DNA sequence analysis. Both complementary strands of the the

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† The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF133700.

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1  The abbreviations used are: NGC, neuroglycan C; EGF, epidermal growth factor; FISH, fluorescence in situ hybridization; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-mediated PCR; bp, base pairs.
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Results

Cloning of the Mouse NGC cDNA—When a cDNA library (500,000 clones) of the mouse brain was screened for the NGC-specific sequence using a rat cDNA (13) as a probe, more than 400 colonies gave a positive signal above background. Plasmid DNAs were prepared from 20 individual colonies, digested with EcoRI and XhoI, and then the identity of the plasmid DNAs was confirmed by Southern hybridization with the rat cDNA used as a probe. The isolated plasmids had a cDNA insert ranging from 2.1 to 2.6 kilobases in size. Most of these cDNA inserts covered the full length of the coding region predicted from the sequence of the rat NGC cDNA.

DNA sequencing of these 20 clones suggested that there existed three isoforms (designated NGC-I, -II, and -III) of NGC in the mouse brain (Fig. 1). Of the 20 clones, sixteen, two, and two encoded NGC-I, NGC-II, and NGC-III, respectively. The core protein of the major isoform, NGC-I, was composed of 539 amino acid residues and had a multidomain structure identical to rat and human NGCs: a signal peptide, an N-terminal domain to which chondroitin sulfate chains might be attached, an acidic amino acid cluster, an EGF-like domain, a membrane-spanning segment, and a C-terminal cytoplasmic domain with two putative phosphorylation sites for protein kinase C in the cytoplasmic domain are indicated by open triangles.

As shown in detail below, only the N-terminal portion of pro-NGC, including the signal peptide, was structurally different between NGC-I and NGC-II, and NGC-III had a peptide insertion (after Asn491) composed of 27 amino acid residues in the cytoplasmic domain of NGC-I (Fig. 1).

Cloning of the Mouse NGC Genomic DNA—The NGC gene had a size of approximately 17 kilobases and was comprised of six exons (Fig. 3). There were two alternative splicing sites. First, exon 1 covered the 5'-untranslated region and a part of the coding region common to NGC-I and -III mRNAs, and exon 1' covered them unique to NGC-II mRNA. Second, exon 5 coded an amino acid sequence (VRKFCDTPRVSSPHARALAHYD-NIVCQ) unique to NGC-III, which represents a part of the cytoplasmic domain with a new putative phosphorylation site.
for protein kinase C. No introns existed between exons 1 and 2 nor between exons 4 and 5. Other exon/intron junctions followed the GT/AG rule (Table I).

Exon 2 encoded the chondroitin sulfate attachment domain, the acidic amino acid cluster, and a part of the EGF-like module containing two cysteine residues. Exon 3 coded the remaining part of the EGF-like module containing four cysteine residues and the transmembrane domain. The cytoplasmic domain was encoded by exons 4 and 6 in the cases of NGC-I and -II and exons 4, 5, and 6 in the case of NGC-III.

Chromosomal Mapping of the Mouse NGC Gene—In 85 cells examined, 142 double-dot signals were observed by FISH using the mouse genomic DNA of NGC as a probe. These signals were specifically located in region F1 of mouse chromosome 9 (Fig. 4). Other locations did not show any double-dot signals. When mouse NGC-I cDNA was used as a probe, FISH signals were observed in the same region (data not shown). Based on these results, the mouse NGC gene was assigned to chromosome 9 at band F1.

Expression of Three Splice Variants of NGC in the Mouse Cerebellum—To confirm the expression of three splice variants coding NGC-I, -II, and -III in the mouse cerebellum, RT-PCR was performed using total RNA preparations of the cerebella from 5-day-old and adult mice. When the part of the NGC cDNA corresponding to exons 2–6 was amplified by RT-PCR, only two products of 408 and 489 bp were observed in both the 5-day-old and adult samples (Fig. 5A). The DNA sequence analyses of these products showed that the smaller one, which was the major product, represented the corresponding part of cDNAs of NGC-I and II, and that the larger one represented the corresponding cDNA fragment of NGC-III with the short peptide insertion.

When the parts of the NGC cDNAs corresponding to exons...

FIG. 2. Amino acid sequence alignment of mouse NGC-I (the major isoform) and rat NGC core proteins. The homology between mouse NGC-I and rat NGC is 94.3%.

**Table I**

| Exon | Exon length | Splice donor | Splice acceptor | Type |
|------|-------------|--------------|-----------------|------|
| 1    | 97          | ACCGGttagg    | cacagCACGG      |      |
| 2    | 1096        | TGCAGgtaac    | tgtagGTGTA      | I    |
| 3    | 189         | ACCAAgtac     | tctagTAAAT      | II   |
| 4    | 76          | CAAAtgtagc    | tccagGACGA      | O    |
| 5    | 81          | TCAAGgtac     | tccagGACGA      | O    |
| 6    | 159         |              |                 |      |

* The number from A of the first exon to the end base of exon 1.  
* The number from the first base of exon 6 to C of the last codon.

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When the parts of the NGC cDNAs corresponding to exons...
1–2 and exons 1’–2 were amplified by RT-PCR, only one product was detected, with a size of 460 bp in the former case (Fig. 5B) and 596 bp in the latter (Fig. 5C), in both the 5-day-old and adult samples. These findings indicate that the three splice variants of NGC are actually expressed in both the immature and mature cerebella of the mouse.

Western Blot Analysis of NGC in the Mouse Cerebellum—Developmental change in the amount of NGC in the mouse cerebellum was examined from day 5 to adulthood by Western blotting using an antiserum raised against the recombinant polypeptide representing the chondroitin sulfate attachment domain of rat NGC (14). NGC was recognized as a smear, which is characteristic of proteoglycan, in homogenates of the 5-day-old mouse cerebellum (Fig. 6A). The average molecular mass of the smear was 150 kDa, the same as that of rat NGC (13). Digestion of the homogenates with chondroitinase ABC produced a relatively narrow band with a molecular mass of 120 kDa. The amounts of the 120-kDa band in the chondroitinase-treated samples decreased gradually with the cerebellar development from day 5 to day 15 and reached an adult level at around 20 days when the cerebellum is almost matured.

Unexpectedly, a significant amount of the 120-kDa band was visible in the cerebellar homogenates of 20-day-old and adult mice even without the chondroitinase treatment. The migration position of this band was not altered by the chondroitinase digestion, showing that NGC occurs in a nonproteoglycan form without chondroitin sulfate chains in the mature cerebellum. In other words, NGC is a part-time proteoglycan.

In contrast, most NGC in the mouse cerebrum existed in a proteoglycan form with a molecular mass of 150 kDa at various postnatal days from 5-day-old to adulthood (Fig. 6B). The NGC content of the cerebrum increased with the postnatal development up to around day 15 and then decreased gradually reaching about the half-level of the peak at adulthood.

Localization of NGC in the Mouse Cerebellum—When the cerebellum of the 5-day-old mouse was immunostained with the anti-NGC antiserum, the Purkinje cell layer was stained diffusely (Fig. 7A). The reaction was very weak. At day 10, the reaction products were observed on the cell bodies and on the first and second (arrowheads) dendrites of the Purkinje cells.
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FIG. 7. Immunohistochemical localization of NGC in the cerebellum of young and adult mice. A, 5-day-old mice. Weak and diffuse staining is observed in the Purkinje cell layer. Arrows indicate the soma of the Purkinje cells. B, postnatal day 10. The soma (arrows) and the first and second dendrites (arrowheads) of the Purkinje cells are immunostained. C, adult mice. Arrows and arrowheads indicate the soma and the dendrites of the Purkinje cells, respectively. D, 10-day-old mice. Section was immunostained after chondroitinase ABC treatment. The soma of the Purkinje cells (arrows) are stained stronger than that without chondroitinase ABC treatment (C, arrows). In the dendrites of the Purkinje cells, strong reactivity is concentrated on the diverging points (arrowheads). E, high magnification of D. Diverging points are markedly immunostained. F, high magnification of C. Arrowheads indicate the immunoproducts on the large dendrites of the Purkinje cells. Sections were cut sagittally and immunostained with the antisera to rat NGC (1:250) and a peroxidase-conjugated anti-rabbit antibody (1:1000). Bar, 25 μm in A–F.

FIG. 8. Localization of NGC in the Purkinje cells of the five-day-old and adult mice. A, five-day-old mice. The cell membrane (large arrows), synaptic junction (asterisks), and the membrane of endoplasmic reticula (small arrows) are immunostained. The membrane of endoplasmic reticula in the other cell is not stained. PN, the nucleus of the Purkinje cell; PS, the soma of the Purkinje cell; SV, synaptic vesicles. Bar, 200 nm. B, adult mice. The hypolemmal cisterna (arrowheads) reacted strongly with anti-NGC antibody. Cell membrane (large arrows) and the membrane of endoplasmic reticula (small arrows) are also immunostained. PD, the dendrite of the Purkinje cell. Bar, 500 nm. C, high magnification of B. The membrane of hypolemmal cisterna (arrowheads) and cell membrane (large arrows) are stained. PD, the dendrite of the Purkinje cell. Bar, 200 nm. Sections were cut sagittally and immunostained with the antisera to rat NGC (1:250) and a peroxidase-conjugated anti-rabbit antibody (1:1000).

DISCUSSION
Three Predicted Isoforms of NGC—In the present work, we showed that three splice variants were expressed from a single gene in the mouse brain (Fig. 3). RT-PCR demonstrated that these variants were actually expressed in both the immature and mature cerebella of the mouse (Fig. 5). The major variant coded a protein (designated NGC-I) composed of 539 amino acid residues, namely the mouse counterpart of NGC that we originally isolated from the developing rat brain (13). The other variants coded NGC isoforms designated as NGC-II and -III.

To determine the translation initiation site for NGC-II, we completely sequenced the first intron between exon 1 and 1'. However, the consensus sequence (A/G)NNATG(A/G) for the initiation of protein synthesis (20) could not be found in this region. Instead, we noticed that the third methionine (126th) in NGC-I was conserved in rat and human NGCs. The initial amino acid of NGC-II may be the third methionine of NGC-I.

NGC-III has a peptide insertion, which neither NGC-I nor NGC-II has in their cytoplasmic domain (Figs. 1 and 3). We are preparing antibodies in the rabbit specific to the peptide composed of the 27 amino acid residues to examine the developmental changes in the amount and location of this isoform in the brain. Preliminary immunohistochemical experiments suggested that one of the peptide antibodies stained, but very faintly, the Purkinje cells in the cerebellum, suggesting that NGC-III is actually expressed as a protein.

The predicted structure of NGC-III is very interesting in terms of protein phosphorylation. NGC-I has two putative phosphorylation sites for protein kinase C in its cytoplasmic domain, and NGC-III has an extra site in the peptide insert. A recombinant peptide representing the cytoplasmic domain of NGC-I can be phosphorylated well by protein kinase C isolated
from the brain. Whether the extra site in the NGC-III is actually phosphorylated in vitro and has some effects on signal transduction system in the cerebellum is open for further investigation.

Proposed Functions of NGC in the Cerebellar Development—The cerebellum is composed of several types of neurons. Of these cells, only Purkinje cells were stained with the antiserum against the chondroitin sulfate attachment domain of NGC. Purkinje cells form synapses with two major fiber systems: the climbing fiber system largely originated from the inferior olivary complex and the parallel fiber system from the cerebellar granule cells. At almost the same stage of the 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 the spiny branchlets of the Purkinje cell dendrites (21). The developmental change in the localization of NGC on the Purkinje cells correlates well with synaptogenesis of the climbing fiber system with the Purkinje cells; NGC deposition and climbing fiber’s synapse buttons are found on the soma of the Purkinje cells at day 5, on both the soma and the large dendrites at day 10, and on the large dendrites in the mature cerebellum (Fig. 7). These findings may suggest that NGC expressed on the Purkinje cells mediates the adhesion and/or synaptogenesis of the climbing fibers with the Purkinje cells. Alternatively, NGC may inhibit the adhesion and/or synaptogenesis of the parallel fibers with the large dendrites of the Purkinje cells.

It is of interest that NGC acts as a typical part-time proteoglycan in the Purkinje cell differentiation; NGC exists in a proteoglycan form with chondroitin sulfate chains on immature Purkinje cells and in a nonproteoglycan form on the large dendrites of mature Purkinje cells. Neither the biological significance nor molecular mechanism for the developmental change in the NGC structure is clear at this moment. However, it may be that the proteoglycan form of NGC on the Purkinje cells exerts more of an attractive effect on climbing fiber adhesion or more of a repulsive effect on parallel fiber adhesion.

As shown in Fig. 7, NGC is concentrated at the hypolemmal cisternae of the Purkinje cell dendrites in the mature cerebellum. The hypolemmal cisterna supplies membrane to the plasma membrane. Strong NGC immunoreactivity is observed on the diverging points of the Purkinje cell dendrites at day 10 (Fig. 7), where membrane supply is needed. Considering these findings, NGC may be related to membrane supply system, especially in latter stage of the cerebellar development.

Recently, Ozaki et al. (22) have suggested that neuregulin-β, a member of the neural EGF family, increases the expression of NR2C, which is a subunit of NMDA receptors by innervation of the glutamatergic mossy fibers to the internal granule cells in the cerebellum. Likewise, by innervation of the climbing fibers to the Purkinje cells, NGC, another member of the neural EGF family, may regulate the expression of some receptors in the cerebellum.

Location of the NGC Gene on Mouse Chromosomes—We have assigned the human NGC gene to chromosome 3p21.3 (14) and the mouse NGC gene to chromosome 9F1 (Fig. 4). The human chromosome 3p21.3 is known to be syntenic to the distal segment of mouse chromosome 9 on the basis of comparative gene mapping (23, 24). The present result reconfirmed the syntenic association between human chromosome 3p21.3 and mouse chromosome 9F1. A computer search revealed that there had been reported no mouse mutants whose responsible genes are supposed to map to chromosomal region 9F1.

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