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Review

Neurocan: a brain chondroitin sulfate proteoglycan

U. Rauch *, K. Feng and X.-H. Zhou

Department of Experimental Pathology, Lund University, 22185 Lund (Sweden), Fax +46 46 158 202,
e-mail: Uwe.Rauch@pat.lu.se

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Abstract. Neurocan is a chondroitin sulfate proteoglycan of the lectican family and a component of the extracellular matrix of the central nervous system. It is mainly expressed during modeling and remodeling stages of this tissue. Neurocan can bind to various structural extracellular matrix components, such as hyaluronan, heparin, tenascin-C and tenascin-R, and the growth and mobility factors FGF-2, HB-GAM, and amphoterin. Neurocan can also interact with several cell surface molecules, such as N-CAM, L1/Ng-CAM, TAG-1/axonin-1, and an N-cadherin-binding N-acetyl-galactosamine-phosphoryl-transferase, and in vitro studies have shown that neurocan is able to modulate the cell-binding and neurite outgrowth promoting activites of these molecules. Current analysis of the molecular structures and substructures involved in homophilic and heterophilic interactions of these molecules and complementary loss-of-function mutations might shed some light on the roles played by neurocan and interacting molecules in the fine tuning of the nervous system.

Key words. Neurocan; proteoglycan; brain; extracellular matrix; glycosaminoglycan; tenascin; neural cell adhesion molecule.

About 10 years ago, with the aid of monoclonal antibody (mAb) 1D1, the brain chondroitin sulfate proteoglycan neurocan was isolated and characterized as a component of the soluble fraction of rat brain proteoglycans [1]. Subsequent analysis of the primary structure revealed that neurocan shares extensive homologies with the cartilage proteoglycan aggrecan [2], with versican, a proteoglycan expressed in a wide variety of tissues [3], and with brevican, another brain proteoglycan [4]. These four molecules constitute the lectican family of hyaluronan-binding proteoglycans, which has been a topic of recent reviews in this [5] and other [6–9] journals.

This review will focus solely on neurocan. It will describe its structural peculiarities, its interactions with other extracellular matrix and cell surface molecules, the developmental and induced expression of the molecule, and also biological activities of neurocan which have been observed in vitro. Finally, the expression of neurocan will be related to the maturation of the extracellular brain matrix and to observations made by interfering with the function and expression of molecules which have been shown to interact with neurocan.

* Corresponding author.

The structure of neurocan

The primary structures of rat [10], mouse [11], human [12], and chicken [13] neurocan are publicly available. Like all lecticans, neurocan is composed of homologous domains built up from common extracellular matrix protein modules, and parts without any obvious homology, a central mucin-like region, and a C-terminal extension (fig. 1). In the mouse neurocan gene, all single or multiple exons representing complete protein modules or mucin-like regions are separated by type I introns [11]. This opens up the possibility that any module may be

spliced out without changing the reading frame, but so far no alternative splice variant of neurocan has been reported. The two homologous domains are, with more than 90% identical amino acids, well conserved among rat, mouse, and human [12], and have about 70% sequence identity even in chicken neurocan, whereas in the central mucin-like region, identity among the mammalian neurocan sequences is less than 50% and, except for the overall length, amino acid composition, and the number of potential glycosaminoglycan attachment sites, little similarity can be observed with the central region of chicken neurocan. Comparatively well conserved, with more than 50% identical amino acids even in chicken, is the C-terminal extension of 42–48 amino acids appended to the C-terminal homologous domain.

The N-terminal domain

The N-terminal domain in neurocan and without exception in all reported lectican isoforms is composed of one immunoglobulin (Ig) module and two link modules (fig. 1). Ig modules are widespread structural components in extracellular molecules, whereas link modules have been observed only in a limited number of usually hyaluronan-binding molecules. In a module-based analysis of the human genome, only 13 molecules with a total number of 23 link modules were identified, in contrast to 381 molecules with 930 Ig modules [14]. Structural investigations by electron-scattering experiments indicated a linear arrangement of the three modules [15]. However, this arrangement is barely compatible with the structure of the link module as determined by nuclear magnetic resonance spectroscopy from the TSG-6 link module. According to this structure, link modules are related to C-type lectin modules and the N and C termini are located side by side, pointing in the same direction [16]. The globular appearance of this domain in electron micrographs [17] suggests a compact, possibly tetrahedral arrangement where the C terminus of the Ig module, both termini of the link modules, and the N terminus of the central region could meet in the center (fig. 1). However, the overall arrangement of subunits of these lectican domains and of the related link proteins remains to be experimentally determined.

The complete N-terminal domain of neurocan can be efficiently expressed and secreted in eukaryotic cells, indicating an independent folding unit [17]. Just as efficiently expressed and secreted are fusion proteins of the N-terminal domain of neurocan with a C-terminal alkaline phosphatase (AP) module which have been used as probes for hyaluronan in histochemical analysis [18]. Surprisingly, in contrast to these observations, the N-terminal domains of versican and aggrecan have been reported to be secreted very inefficiently [19, 20].

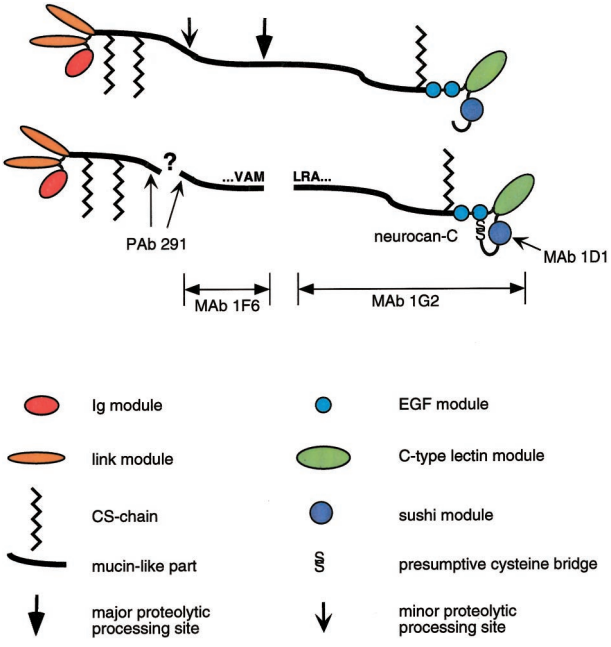

Figure 1. Proposed domain organization, proteolytic processing, and position of antibody epitopes in rat neurocan. The presumptive C-terminal amino acids of the 130-kDa N-terminal fragment and the determined N-terminal amino acids of the 150-kDa C-terminal fragment, starting with leucine 639, are indicated. Ig, immunoglobulin-like; EGF, epidermal growth factor-like; CS, chondroitin sulfate.

The central region

The about 600 amino acids of the central region of neurocan are characterized by a high content of serine, threonine, and proline, and a lack of cysteine residues, except for one cysteine residue in the chicken sequence. This part of the molecule is substituted with about three chondroitin sulfate chains mainly sulfated in the 4-position of the GalNAc residues.These chains have a median size of 22 kDa and 20% chondroitin 6-sulfate in the brain of 7-day-old rats, and a median size of 32 kDa and less than 3% chondroitin 6-sulfate in the brain of adult rats. Due to up to 40 additional O-linked oligosaccharides, the central region has, on electronmicrographs, a mucin-like extended appearance with an average length of 70 nm connecting terminal globular domains. Thus, about nine amino acids have to account for every extended nanometer. However, the formation of subdomains with a defined structural arrangement in this region is not excluded, since in other extended molecules like mucins [21], or the central region of aggrecan, the protein backbone can be stretched even further, to only four amino acids per nanometer on average.

In the mouse genome, the central region of neurocan is encoded by two exons of 597 and 1209 nucleotides [11]. Comparison of the very similar rodent amino acid sequences encoded by those exons with the human sequence reveals that the sequence encoded by the first

exon is (with 57% identity) much better conserved than the sequence encoded by the second exon (with 29% identity) [12]. A major contribution to the better conservation of the first, shorter part of the central region comes from well conserved sequences around potential glycosaminoglycan attachment sites, in particular those sites in the vicinity of the globular domains. This presence of glycosaminoglycan attachment sites in positions close to the globular domains is characteristic of neurocan, and not conserved in all lecticans. There is evidence that attached glycosaminoglycan chains modulate interactions of the C-terminal globular domain (see below) [22], but not the N-terminal domain [13]. Interestingly, the occurrence of glycosaminoglycan free N-terminal hyaluronan-binding fragments of neurocan in vivo has never been observed (or reported), whereas N-terminal domains devoid of any glycosaminoglycan chains are known proteolytic processing products from aggrecan [23], versican [24], and brevican [25].

One of the more conserved parts of the central regions of the mammalian neurocans, with 14 out of 20 amino acids identical, is also the sequence surrounding the major proteolytic cleavage site of neurocan in rat brain, where, during brain development, neurocan is increasingly cleaved into an N-terminal and C-terminal fragment with core proteins of 130 and 150 kDa, respectively [1, 26] (fig. 1). The exact location of a minor proteolytic cleavage site responsible for the generation of an N-terminal neurocan fragment with a core protein of about 90 kDa which has been observed in rat brain neurocan preparations [27] has not been reported.

The C-terminal domain

The globular C-terminal domain of neurocan consists of two epidermal growth factor-like (EGF) modules, a C-type lectin module, a sushi module, and a C-terminal extension of about 45 amino acids (fig. 1). All modules are common components of extracellular molecules or extracellular parts of membrane proteins [14], and are probably able to fold independently. Thus, in aggrecan, the EGF and sushi module can be alternatively spliced [2], and the C-type lectin domain of all four lecticans can be independently secreted by mammalian cells [28]. Moreover, secreted proteoglycans can be observed in the medium of mammalian cells transfected with neurocan cDNAs from which the lectin module and the second EGF module have been eliminated, or which have been truncated either after the C-type lectin or the sushi module [29]. The second EGF module shows characteristics of calcium-binding EGF modules, and its stability and ability to bind calcium might depend on the integrity of the sequence connecting it to the first EGF module, since in similar tandemly arranged EGF modules, those amino acids contribute to the binding of calcium [30, 31].

Among the lecticans, one peculiarity of the C-terminal neurocan domain in rodents and humans, but not chicken, is the occurrence of one additional cysteine in the second EGF module and a second additional cysteine at the very end of the sequence, which might form another cysteine bridge, in addition to the intramodular cysteine connections (fig. 1). Another peculiarity is the accumulation of basic amino acids and histidines in the C-terminal extension, a feature which is even conserved in chicken neurocan. Within the C-terminal extension, those basic residues create potential furin recognition sites. However, in SDS-PAGE, recombinantly expressed C-terminal rat neurocan domains starting with amino acid 925 (fragment D925) [17] with and without the two additional cysteines mutated to alanines under reducing as well as nonreducing conditions showed no obvious differences in their migration behavior, and N-terminal sequencing of unmutated fragment D925 after SDS-PAGE separation under nonreducing conditions revealed only one N-terminal sequence, representing the expected N terminus after cleavage of the signal peptide [K. Mann and U. Rauch, unpublished observation]. Thus, these experiments revealed no evidence for a disulfide bridge or proteolytic cleavage.

Interactions

Interactions with glycosaminoglycans

Based on the sequence homology to other hyaluronan-binding molecules, an interaction of the N-terminal neurocan domain with hyaluronan was predicted and could be demonstrated by cochromatography of this fragment with hyaluronan during gel permeation chromatography [17] and the binding of AP-tagged or His-tagged N-terminal neurocan domains to hyaluronan adsorbed to microtiter plates [U. Rauch and T. Kalkowsky, unpublished observation] (fig. 2). Since link protein has been identified with the monoclonal antibody 8A4 in neurocan preparations isolated from rat brain [27], it is tempting to speculate that there are ternary complexes of hyaluronan, link protein, and proteoglycan in brain, as in cartilage. Recently, the existence of a brain-specific homologue, BRAL1, of the classical cartilage link protein has been reported [32], and expressed sequence tags coding for link protein-like sequences indicate the presence of even more link protein homologues [T. Oohashi, personal communication]. However, since the monoclonal antibody 8A4 does not recognize BRAL1 [U. Rauch and S. Hirakawa, unpublished observation], the classical cartilage link protein may be involved in the formation of proteoglycan aggregates even in brain (fig. 2). This, however, remains to be demonstrated.

Under physiological buffering conditions, heparin binds to neurocan in its native, glycosaminoglycosylated form

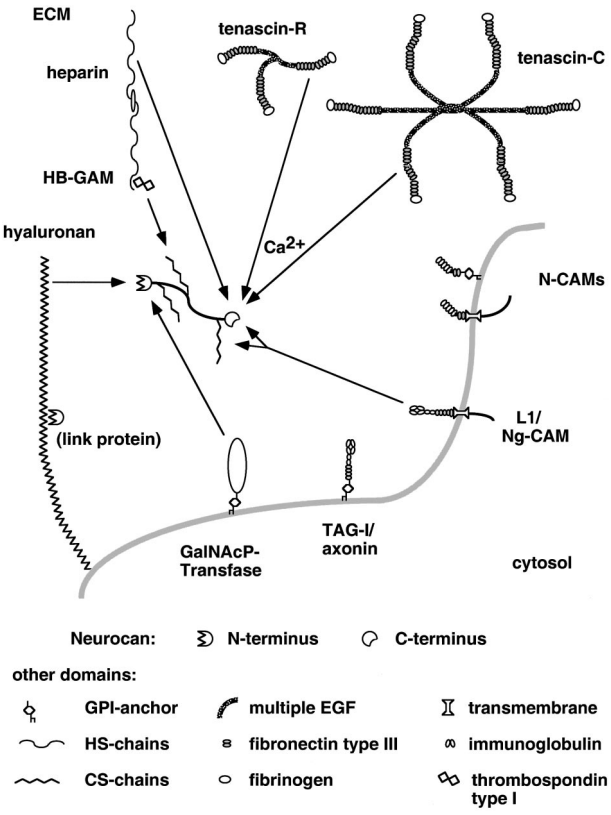

Figure 2. A scheme reflecting the observed or presumptive shapes of various ligands of neurocan. Shapes of neurocan [17], tenascin-C [40], tenascin-R [45], and N-CAM [120] are from rotary shadowing electron microscopic analysis; shapes of L1/Ng-CAM and TAG-1/axonin-1 are inferred from the crystal structure of the first four Ig modules of axonin-1 [61]. Arrows from ligands point to parts of neurocan which are completely or partially involved in the respective interactions. ECM, extracellular matrix; GPI, glycosylphosphatidyl inositol; HS, heparan sulfate; CS, chondroitin sulfate; EGF, epidermal growth factor-like.

[22]. The interaction can be mediated by the C-terminal globular domain of neurocan (fig. 2), and is enhanced in the absence of neurocan-linked chondroitin sulfate chains. However, since the occurrence of chondroitin sulfate-free neurocan molecules has never been observed, and neurocan can be considered to be a full-time proteoglycan, the physiological relevance of this observation is not clear. Interestingly, various other molecules which bind to neurocan (see below) also interact with heparin. Whereas the interaction of neurocan with tenascin-C appears to be stabilized by heparin [22], other neurocan-binding molecules, especially those which show a reduced interaction with the neurocan core protein after removal of the chondroitin sulfate chains, might be compromised in their ability to bind to neurocan in the presence of heparin or other highly charged heparan sulfate chains.

Interactions with growth and mobility factors

Interactions of neurocan with the growth and mobility factors FGF-2 [33], HB-GAM, and amphoterin [34], all of which can bind to heparin, have been described. FGF-2 is a 16- to 25-kDa growth and differentiation factor for a variety of cell types, widely distributed in nervous tissue. Binding of neurocan to FGF-2 is reduced by 35% after chondroitinase treatment of the proteoglycan. For the neurocan core protein, a dissociation constant of 12 nM was determined from Scatchard plots of radioligand-binding saturation curves [33].

HB-GAM is a neurite-promoting 18-kDa matrix-associated protein lining growing axons in brain. It is composed of two thrombospondin type I modules, a module which has been observed in various other extracellular matrix and cell surface molecules [35] (fig. 2). For the interaction with neurocan, a dissociation constant of 1 nM was determined from Scatchard plots of radiolabeled ligand-binding saturation curves [34]. Amphoterin is a 30-kDa protein found in the leading edge and in substrate-attached material of growth cones and migrating cells. Like FGF-2, amphoterin lacks a classical secretion signal. However, when supplied extracellularly, it strongly promotes neurite extension [35]. The dissociation constant determined for neurocan and amphoterin was 8 nM [34]. In contrast to the interaction with FGF-2, the interaction of neurocan with HB-GAM and amphoterin appears to occur in a mainly chondroitin sulfate chain-dependent manner. Chondroitinase treatment of neurocan reduced the binding to HB-GAM and amphoterin by 80% and the presence of free chondroitin sulfate chains reduced the binding of those molecules to neurocan by 35–45% [34]. Since neurocan-linked chondroitin sulfate chains show decreasing chondroitin 6-sulfate content during neurodevelopment [1], it is interesting to note that HB-GAM-induced migration of neurons could be inhibited by addition of chondroitin 6-sulfate chains, but not by addition of chondroitin 4-sulfate chains [36].

Interactions with structural matrix proteins

Neurocan has been shown to bind to the two related oligomeric extracellular matrix proteins tenascin-C [37] and tenascin-R [34] (fig. 2). Tenascin-C is a hexameric glycoprotein assembled from six identical subunits. Each subunit is composed of an N-terminal hexamerization domain, 14 EGF modules, 8–17 fibronectin type III modules, and a C-terminal globular fibrinogen module [38, 39]. Observed by rotary shadowing electron microscopy, the length of each of the six subunits extending from a central knot is similar to the overall length of neurocan [40]. The dissociation constants for the binding of neurocan and the neurocan C-terminal domain to tenascin-C, obtained by BIAcore binding studies, are 3–4 nM and 17 nM, respectively [41]. The same dissociation constant

was observed in radioligand-binding assays with the entire neurocan molecule [37]. Radioligand-binding studies performed with tenascin-C fragments and deletion mutants pointed to a prominent interaction of rat brain-derived neurocan with the C-terminal fibrinogen module of tenascin-C [42], whereas a prominent retardation of a tenascin-C fragment comprising the fourth and fifth fibronectin type III domain could be observed by affinity chromatography with an immobilized recombinantly expressed C-terminal neurocan domain [41]. This interaction could be enhanced by the addition of heparin [22]. From its molecular dimensions, its affinity, and its spatial and temporal expression patterns, tenascin-C appears to offer a perfect partner for neurocan in the assembly of glycoprotein/proteoglycan/hyaluronan aggregates which have been proposed as organizers of the extracellular matrix in the developing central nervous system [43].

In contrast to tenascin-C, the temporal expression pattern of tenascin-R, which is expressed in increasing amounts during development, is inversely related to the predominantly declining postnatal expression of neurocan. Tenascin-R is a homotrimeric glycoprotein composed of an N-terminal oligomerization domain, four EGF-modules, eight to nine fibronectin type III modules, and a C-terminal globular fibrinogen module [44]. By rotary shadowing electron microscopy, the length of each subunit is considerably smaller than the overall length of neurocan [45]. In radioligand-binding assays, the dissociation constant of neurocan to tenascin-R was 7 nM, i.e., the same order of magnitude as for the binding of neurocan to tenascin-C. In BIAcore binding studies for the binding of just the C-type lectin domain of neurocan with fibronectin type III modules 3–5 of tenascin-R, a dissociation constant of 31 nM was measured [46], indicating participation of this domain together with other parts of the molecule in the interaction. Interactions of tenascin-R, direct or indirect, with the N-terminal part of neurocan appear to be important for the localization of this neurocan fragment in perineuronal nets, since the presence of N-terminal neurocan fragments in those structures is significantly reduced in tenascin-R knockout mice [47].

Interactions with neural cell adhesion molecules

N-CAM, L1/Ng-CAM, and TAG-1/axonin-1 are homophilically interacting cell adhesion molecules and members of the Ig superfamily believed to be involved in neurite outgrowth, fasciculation, and navigation [48]. N-CAM can occur in various splice variants, among them type I transmembrane and glycosylphosphatidyl inositol (GPI)-anchored forms [49] (fig. 2). The extracellular domain consists generally of five distal Ig modules and two membrane proximal fibronectin type III modules. There is evidence for an involvement of the three N-terminal Ig modules in the homophilic interaction of N-CAM [50, 51], for the fourth in heterophilic binding to oligosaccharides [52], and for the fifth in polysialylation [53] of the molecule. The homophilic interaction can be inhibited by neurocan derived from rat brain [54], and by various recombinant neurocan fragments not overlapping in their primary structure [55] in Covasphere aggregation assays. A common characteristic of all neurocan fragments with inhibitory activity is the presence of one of the two globular domains of neurocan, combined with an extended component, either a substantial part of the central region of neurocan, or a glycosaminoglycan chain. Each of these components by itself has no inhibitory effect in the aggregation assay, although binding of N-CAM to some of them could be detected in BIAcore and solid-phase binding studies. Binding of N-CAM to the immobilized chondroitinase-treated complete neurocan core protein gave a dissociation constant of 28 nM in BIAcore binding studies [55], whereas radioligand-binding studies with untreated neurocan proteoglycans revealed a stronger interaction, with a dissociation constant of 0.4 nM [56]. In those radioligand-binding studies, chondroitinase treatment of neurocan reduced the number of molecules which were still able to bind to N-CAM to about 20%, and combined with heat treatment, to nearly background levels. Concentration-dependent inhibition of neurocan binding to N-CAM was produced by aggrecan, a cartilage proteoglycan with a high concentration of chondroitin sulfate, and by free chondroitin sulfate chains with a disaccharide composition similar to neurocan from early postnatal brain [56]. All these data show that chondroitin sulfate chains are strongly involved in the binding of neurocan to N-CAM, but the core protein of neurocan retains binding activity even in their absence.

Similar general characteristics were observed for the interaction of neurocan with Ng-CAM, the presumed chicken homologue of mammalian L1. In radioligand-binding assays, a dissociation constant of 0.7 nM, a 70% reduction of binding after chondroitinase treatment, and an inhibition of the interaction by chondroitin sulfate structures was observed [56]. Neurocan also inhibits the homophilic binding of Ng-CAM [54]. L1/Ng-CAM occurs only as a type I transmembrane molecule and exhibits much less variability in splice variants than N-CAM [57]. The extracellular domain consists of six Ig modules and five fibronectin type III modules (fig. 2), and the cytoplasmic part of L1 is anchored to the actin cytoskeleton via ankyrin. In humans, mutations in L1, which is located on the X chromosome, cause abnormal brain development and mental retardation [57]. Studies with recombinant rat neurocan and human L1 fragments indicated that the N-terminal Ig module of L1 is responsible for the interaction with neurocan [58]. Interestingly, Fc fusion proteins of the first L1 Ig module containing one particular mutation, L120V, have a reduced ability to serve as substrate for the attachment of T cells

[59], a process mediated by neurocan, which is naturally expressed by those cells [58]. This mutation, which causes all typical symptoms of L1 mutations (enlarged ventricles, mental deficits, spastic paraplegia, and deformed thumbs), showed no effect in homophilic or other heterophilic L1 interaction studies [60]. On the neurocan side, the binding to L1 appears to depend on a cooperative interaction of a chondroitin sulfate chain and the sushi module in the neurocan C-terminal domain [59] (fig. 3).

Axonin-1, the presumed chicken homologue of rat TAG-1, is composed of six Ig modules and four fibronectin type III modules. Thus, the structure of the extracellular part of axonin-1 is more similar to NgCAM/L1 than to N-CAM. However, in common with certain N-CAM variants but unlike Ng-CAM/L1, axonin-1/TAG-1 is linked to the cell membrane by a GPI-anchor [48]. The crystal structure of the first four Ig modules of axonin has been solved, and shows a U-shaped structure with contacts between Ig modules 1 and 4, and modules 2 and 3 [61] (figs 2, 3). A similar structure has also been observed for the first four Ig modules of hemolin [62], and can be assumed for the first four Ng-CAM/L1 Ig modules, whereas the arrangement of the N-CAM Ig modules appears to be different [51]. Binding of neurocan to axonin-1 observed in radioligand-binding assays with a dissociation constant of 0.3 mM is in the same range as the affinities to N-CAM and Ng-CAM/L1. However, in contrast to those interactions, the interaction with axonin is independent of the presence of neurocan-linked chondroitin sulfate chains [63].

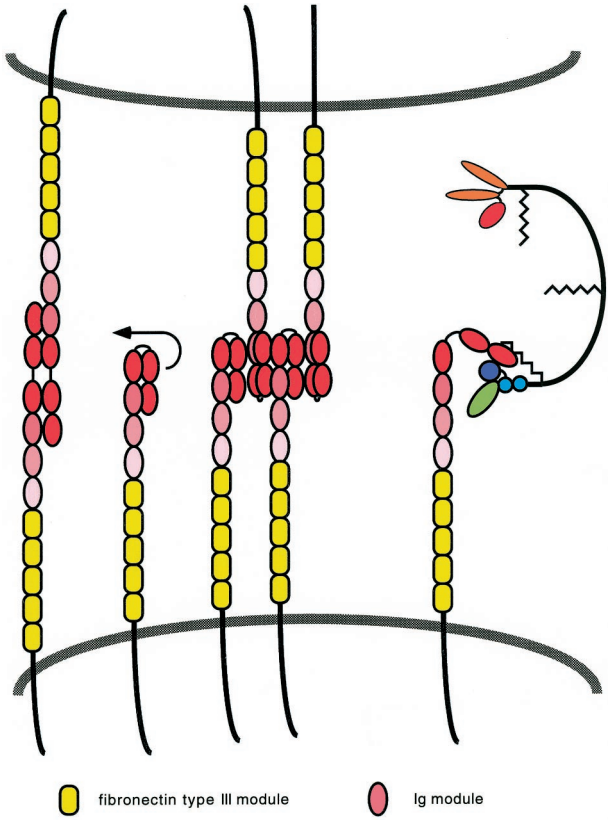

Figure 3. Models of homophilically interacting L1 molecules and the inhibition by neurocan. L1 molecules could interact by an antiparallel alignment of linear-arranged Ig modules which in single molecules fold into a horseshoe-like structure [62] or in zipper-like arrays of alternating antiparallel horseshoe-like structures [61]. In both models, binding of neurocan via the sushi module and a chondroitin sulfate chain to the first Ig module could interfere with the homophilic interaction of L1.

Interactions with other molecules

Another GPI-linked membrane molecule which probably interacts directly with neurocan is N-acetyl-galactosaminyl-phosphoryl-transferase (GalNAcPTase) [64]. Binding of neurocan to GalNAcPTase which specifically associates with N- and E-cadherins initiates a signal that results in coordinate inhibition of N-cadherin and beta-1 integrin function [13]. The interaction with the GalNAcPTase is not dependent on chondroitin sulfate chains, and is apparently independent of any glycosylation of neurocan, since the inhibition of N-cadherin-mediated adhesion can be mediated by bacterially expressed neurocan or neurocan fragments representing the hyaluronan-binding N-terminal domain [13] (fig. 2).

The C-type lectin domain of neurocan has been shown to bind to sulfatide, a sulfated glycolipid which is a major component of myelin expressed in oligodendrocytes and Schwann cells in culture [65].

Expression

Expression of neurocan mRNA

Northern analysis of various adult rat (kidney, brain, lung, liver, muscle) and human (heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas) tissues and human brain substructures (amygdala, caudate nucleus, corpus callosum, hippocampus, hypothalamus, substantia nigra, subthalamic nucleus, and thalamus) indicates that neurocan is only expressed in brain, but there it could be detected in every human brain substructure which was tested [10, 12].

A developmental analysis of neurocan mRNA expression in rat brain [66] showed at embryonic day 13 (E13), the presence of neurocan mRNA in the entire ganglionic eminence and all around the lateral ventricles. During further embryonic development, neurocan mRNA is continuously expressed in all periventricular areas, at E16, apparently still almost homogeneously, but toward the end of embryonic development, more intensely in particular zones (fig. 4). In addition, the message is observed in the ependymal and mantle layers of the spinal cord [66]. In the mouse embryo, at E16, neurocan mRNA is also ob-

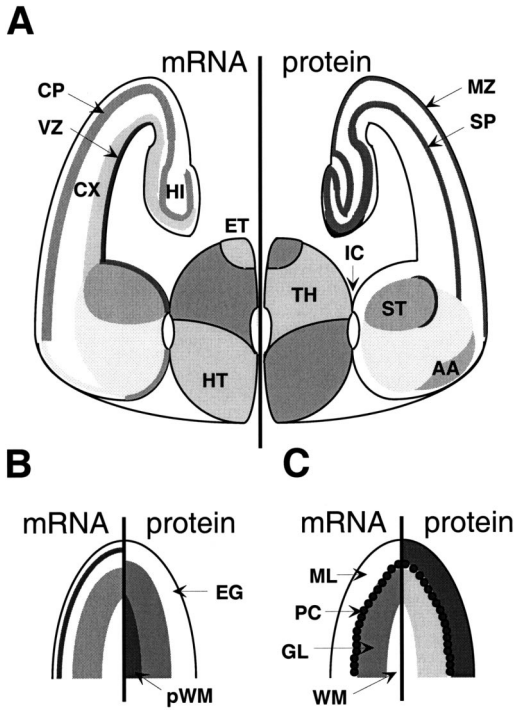

Figure 4. Diagram summarizing in situ hybridization and immunolocalization data from rat brain during late embryonic developmental stages of the forebrain (*A*), and from 1 week postnatal (*B*) and adult (*C*) cerebellum. In situ hybridization data are depicted according to Engel et al. [66], and immunolocalization data according to other studies [69, 71–75]. Note that the observed distribution of neurocan mRNA and protein is sometimes complementary. AA, amygdaloid areas; CP, cortical plate; CX, cerebral cortex; ET, epithalamus; HI, hippocampus; HT, hypothalamus; IC, internal capsule; MZ, marginal zone; SP, subplate; ST, striatum; TH, thalamus; VZ, ventricular zone; EG, external granule layer; pWM, presumptive white matter; GL, granule layer; ML, molecular layer; PC, Purkinje cells; WM, white matter.

served in the eye [M. Moser and X. Zhou, unpulished observation]. In the early postnatal brain, the mRNA of neurocan is still similarly located in the cerebral cortex, the granular layer of the hippocampal formation, the caudate putamen, the septal area, and the thalamic nuclei. In 7-day-postnatal and adult rat cerebellum, neurocan mRNA is differentially expressed according to the neuronal layer (fig. 4), and in addition, in the neurons of the deep cerebellar nuclei [66].

Detection of the neurocan protein in brain

Evidence that neurocan protein is present in rat brain from E12 has been obtained by Western blotting with mAb 1G2 which recognizes an epitope in the 150-kDa C-terminal part of neurocan [67] (fig. 1). This study showed an increasing concentration of neurocan in rat brain during embryonic development to a maximal level at about 1 week after birth, and a consecutive decline in concentration with a concomitant increase in proteolytic processing of the molecule [67]. Corroborative results were obtained by Western blot with the mAb 1D1 [1], which probably recognizes an epitope in the sushi domain (fig. 1), and in dot-blotting experiments with two polyclonal antisera raised against N- and C-terminal parts of the central mucin-like region of neurocan [68].

On E13, the protein of rat neurocan was detected immunohistochemically using mAb 1F6 in the pia-arachnoid and the mesenchyme of the future spinal canal, but not in the roof plate [69]. It should be noted that analysis of neurocan-hyaluronan aggregate affinity isolated with this antibody from rat brain [27] indicates that mAb 1F6 detects the 130-kDa, but not the 90-kDa N-terminal fragment of neurocan. This maps the epitope to a 40-kDa fragment of the central region of neurocan (fig. 1), which might have a higher tissue turnover rate than other parts of the molecule. mAb 1G2 shows immunoreactivity in the roof plate at E13.5 [70]. The neurocan distribution patterns observed during further embryonic development with mAb 1F6, 1G2, and 1D1 in the cortex, hippocampus, striatum, and amygdala [67, 69, 71–74] and with a polyclonal antiserum against neurocan in the diencephalon [75] have shown only gradual changes and are summarized in figure 4. At E19, mAb 1F6 detects neurocan in the region of developing ventral motor neurons, but by then it is absent from the mesenchyme surrounding the spinal cord [69].

At birth, neurocan shows a characteristic distribution in the cerebral barrel field marking the barrel hollows which are free of mAb 1G2 immunoreactivity [76]. After birth, this pattern is downregulated up to postnatal day 7 (P7), and even further at P14. A similar early postnatally upregulated mosaic pattern of neurocan in the striatum appears most prominently at P7–8. In 2-week-old rats, the striatal mAb 1F6 immunoreactivity becomes more evenly distributed again, and this pattern is retained until adulthood [74]. With mAb 1F6, neurocan is detected on P7 in the matrix surrounding the cells in the cortex, and the molecular layer of the hippocampal formation, but not in its external granular cell layer [69]. In P7 rat cerebellum, neurocan is mainly detected in the deeper layers (fig. 4), and in the neurons of the deep cerebellar nuclei [69].

In adult rat brain, different staining patterns with antibodies detecting N- and C-terminal neurocan epitopes (fig. 1) were sometimes evident. In the cerebellum, neurocan is mainly seen in the molecular layer and in the Purkinje cells with mAb 1F6 [69] (fig. 4), whereas it was not observed in the Purkinje cells with mAb 1D1 [1]. In 4-week-old mouse cerebellum, using a polyclonal antiserum against full-length neurocan, signal is distributed mainly in the granular layer and weakly in the molecular layer, but is absent in the Purkinje cells [X. Zhou, unpublished observation]. A diffuse distribution in the cortex, the striatum, and the hippocampal formation is detected

by the polyclonal antiserum 291 recognizing the N-terminal neurocan fragment (fig. 1) (raised against amino acids 483–498 of the rat neurocan sequence) and mAb 1G2 [26, 77]. Polyclonal antiserum 291, but not mAb 1G2, stained the cell surfaces of some neurons in the cortex in a characteristic pattern. The confocal image showed that the N-terminal neurocan fragment is expressed around the cell bodies, the distant part of the dendrites of some of the neurons, and around certain neurons near the synapses, but not at the synapses themselves. The 291 signal visualized by immunoelectron microscopy is localized in the cytoplasm of the glial cell processes encompassing the cell bodies of certain neurons, so-called perineuronal nets [77]. Immunoelectron microscopic studies using polyclonal antibody 291 showed the first perineuronal expression around the first postnatal month, and the number of positive cells increased during brain maturation, although the immunoactivity shown by mAb 1G2 decreased [67]. This difference in the expression pattern detected by polyclonal antibody 291 and mAb 1G2 may be due to distinct interactions and turnover rates of the respective neurocan fragments.

Neurocan in sensory organs

In the rat embryonic retina, neurocan protein was homogeneously distributed, predominantly in the inner aspects from E14 to E16 as determined by the mAb 1G2 [78]. Confocal microscopy showed that neurocan is mainly localized in the extracellular matrix of the retina at these stages. On the other hand, no neurocan signal was detectable in eye at E16 by mAb 1F6 [69]. At E18–P3, the positive signal of neurocan detected by mAb 1G2 was mainly present in the nerve fiber layer (NFL) and the ganglion cell layer (GCL). Using the same antibody, the positive neurocan signal was predominantly present in the inner plexiform layer (IPL), and could also be seen in the outer plexiform layer (OPL) on P7–P14. On P21, the expression level of neurocan in the IPL decreased compared with the level on P14. On P42, neurocan was barely detected in the retina [78]. In chicken, neurocan is observed by E7 in the developing IPL and continues to be associated with this layer through E18 until hatching [13]. Neurocan also appears transiently in the NFL and the OPL between E15–E18 [13].

A polyclonal antiserum against the N-terminal fragment of neurocan showed immunopositive signals in olfactory neuroepithelium and bulb in the developing and adult mouse [79]. Neurocan is present in the perikarya of the primary neurons in the olfactory neuroepithelium from E11.5 onwards, and at E12.5 is observed in the axons of the olfactory bulb, which will form the NFL during embryonic development. At E20.5, the strong immunoactivity of neurocan is also present in the terminal arbors of olfactory axons and the granular layer. From this stage on to P3, neurocan is increasingly distributed in the neuropil of the outer external plexiform layer. Neurocan is expressed in the olfactory neuroepithelium and in the nerve fibers, and weakly present in the glomerular layers of the olfactory bulb in adult mice [79].

Expression of neurocan in nonneural tissues

In chicken embryo, neurocan was expressed on E10 in the brain, heart, heart-forming area, posterior half of the most anterior sclerotomes, the basement membranes surrounding the next group of somites, and the lateral plate mesoderm, as revealed in whole-mount immunostaining (without naming the antibody) [80]. Neurocan was also expressed by T-cell hybridoma cells and activated mouse T lymphocytes [58].

Induced expression of neurocan

In addition to its normal expression pattern, in several cases, neurocan expression has also been induced in astrocytes. Neurocan is produced more abundantly by cultured astrocytes than by cultured neurons [67]. Expression of neurocan by activated astrocytes has been observed in the outer molecular layer of the dentate gyrus after producing entorhinal cortex lesions [81], and in glial scars after cortical injury [82, 83]. In addition, neurocan has been found associated with retinal Mueller cells after transient retinal ischemia [84], and in the immediate vicinity of the lesion site after postcommissural fornix transection [C. Stichel and U. Rauch, unpublished observation]. One characteristic feature of these cases of neurocan reexpression is the occurrence of remodeling processes at the respective sites. The observation that mice lacking neurocan show mild defects in late potentiations of synaptic transmission in the hippocampus [H. Matthies, C. Seidenbecher, R. Faessler and X. Zhou, unpublished observation] might reflect an involvement of neurocan in the remodeling of hippocampal synaptic connections which have been implicated in learning- and memory-related changes in synaptic transmission [85].

Expression and processing of neurocan in vitro

In the supernatant of cultured astrocytes and O-2A lineage cells, neurocan could be observed in its full-length and proteolytically processed form [83]. This indicates an endogenous processing of neurocan to fragments of the size observed in brain. The processing occurred either in astrocyte-conditioned medium or, since the supplementation of the medium with various protease inhibitors had no effect on the processing of neurocan, in the secretory compartments of the astrocytes [83]. The expression level of neurocan could be influenced by various cytokines, with transforming growth factor (TGF)-β and epidermal

growth factor (EGF) exhibiting a positive effect, and interleukin-1, interferon (IFN)-γ and tumor necrosis factor (TNF)-α showing a negative effect [83]. Proteolytic processing of neurocan in N- and C-terminal fragments can also be observed in the course of the recombinant production of neurocan and neurocan fusion proteins, especially after long-term storage of frozen conditioned serum-free medium. N-terminal sequencing revealed that C-terminal fragments started with leucine 639 [K. Mann and U. Rauch, unpublished observations], the same amino acid found at the N terminus of the C-terminal endogenous processing product isolated from rat brain (fig. 1).

In vitro activities

Various studies have shown that neurocan can modify the behavior of neuronal and nonneuronal cells in vitro. Although attachment of neurons and other cells to neurocan can be induced by centrifugation [54, 86], under the force of normal gravity, cells generally do not attach to neurocan. Therefore, to study activities of neurocan in vitro, the ability of neurocan to modulate the binding of neurons or other cells to more adhesive molecules or other substrates was investigated.

Nine-day-old chick embryo brain neurons attach strongly to Ng-CAM, the chicken homologue of L1, adsorbed to polystyrene dishes. This attachment, and the extension of neurites on the L1 substrate, could be inhibited by adsorbing neurocan isolated from rat brain as a second molecule to an Ng-CAM-coated surface [56]. Inhibitory activity was also observed with the endogenous C-terminal processing product of neurocan, neurocan-C (fig. 1). In both cases, inhibition of attachment and neurite outgrowth was not observed by reversing the order, i.e., when first neurocan or neurocan-C and then Ng-CAM were adsorbed to the culture dish. Nine-day-old chick embryo brain neurons also attached well and extended neurites on surfaces coated with anti-Ng-CAM antibodies. This activity could also be inhibited by neurocan. However, when neurocan was adsorbed first to the culture dish, consecutively added anti-Ng-CAM antibodies were not able to reverse the inhibitory effect of neurocan [56]. This observation indicates that the anti-inhibitory activity of Ng-CAM for neurocan-coated surfaces is due to a direct interaction of Ng-CAM with the coated neurocan and neurocan-C molecules. These findings are also in line with recent indications that the sushi module located within the C-terminal neurocan domain is important for the interaction of neurocan with L1 [59].

Such studies suggest that neurocan inhibits L1/Ng-CAM-mediated cell adhesion and axonal outgrowth events, which can be overcome by an excess of L1/NgCAM molecules, or their soluble extracellular domains generated by proteolytic shedding from the cell surface [87]. In vivo, neurons and their processes migrate in some instances across regions of tissue that express high levels of neurocan and L1/Ng-CAM, for example radially migrating neurons in the cerebellum [56], and thalamocortical afferents in the subplate [71]. Depending on the ratio of neurocan and L1/Ng-CAM concentrations, neurocan might reduce the rate of growth cone advance, thereby allowing growth cones more opportunities to sample the environment for directional cues [88].

When olfactory neuroepithelial cells were grown on a substrate of Matrigel combined with recombinant neurocan, a fourfold increase in neuritic growth was observed compared with control cultures on Matrigel alone [79]. The same effect was observed when neurocan was added as soluble protein. This increase was due to a 2.5-fold increase in neurite number and a 1.5-fold increase in the length of the neurites. In the presence of neurocan, neurons formed larger aggregates than on Matrigel alone [79].

Again inhibitory activity of neurocan was observed for the attachment and neurite extension of neuronal cells on N-cadherin [64] which, like L1/Ng-CAM, is a homophilically interacting cell adhesion molecule [89]. In these studies, for which E7 chick embryo neural retina cells were used, neurocan core proteins were equally potent [64], and inhibitory activity was observed even with a chicken neurocan fragment recombinantly expressed in bacteria [13]. This bacterial fragment covered the N-terminal neurocan domain, whereas both molecules which showed inhibitory activity in the Ng-CAM-mediated attachment contained the C-terminal, and only one the N-terminal neurocan domain. Neurocan and the bacterial N-terminal fragment also had an inhibitory effect on the binding of E8 chick retina cells to laminin. This binding was reduced by about 60%, similar to observations with inhibitory anti-β_1-integrin antibodies. In both cases, the inhibitory effects could be eliminated by treatment of the neural retinal cells with phosphatidyl inositol phospholipase C indicating that a GPI-linked molecule is involved in the neurocan-induced effects, which can be seen as a coordinated inhibition of N-cadherin and β_1-integrin-mediated adhesion and neurite outgrowth [13]. This GPI-linked molecule which can probably directly interact with neurocan and with N-cadherin has been found to be a GalNAcPTase. The expression of this enzyme in the GCL and inner nuclear layer of the retina and the expression of neurocan in the intermediate internal plexiform layer led to the suggestion that, neurocan may act as a barrier to neurite extension in the developing retina [13].

In contrast to neural retinal cells, the adhesion of C6 glioma cells to laminin or tenascin-C could not be inhibited by neurocan [37]. In other studies, a mainly neurocan-containing chicken brain chondroitin sulfate proteoglycan fraction significantly inhibited the attachment of fibroblasts to fibronectin and vitronectin, when those

adhesion molecules were mixed with the proteoglycan preparation before they were coated, whereas the adhesion to collagen I was only marginally affected [86]. In these experiments, cells were exposed to a prepared matrix. By serendipity, a matrix endogenously produced by cultured cells themselves was seen to demonstrate neurocan-dependent antiadhesive properties, but only when neurocan was already present during the deposition of the matrix [29]. Neurocan-secreting human embryonic kidney (HEK) 293 cells cultivated in 10% fetal bovine serum-containing medium behave differently. Whereas normal cells grow in monolayers and stay attached to the culture dish for several days, cells secreting neurocan soon start to avoid contact with the conditioned dish surface by growing on top of each other, and finally detach as floating spheroids [29]. This effect is due to the matrix produced and deposited by those cells on the culture dish surface, since untransfected cells on conditioned surfaces show the same behavior, unless the surfaces have been treated with chondroitinase. This effect could only be observed with neurocan or neurocan fragments containing the complete C-terminal domain of the molecule, whereas secretion of neurocan fragments modified with chondroitin sulfate chains, but missing the C-terminal domain or even just parts of it, permitted the growth of cells secreting those molecules into monolayers [29].

These observations point to a general role for the core protein in efficiently immobilizing the chondroitin sulfate chains in a specific location inhibiting attachment and spreading of cells. The observation of these effects in the presence of considerable amounts of nonimmobilized proteoglycans in the medium points to a steric mechanism rather than a specific immobilization of antiadhesive factors by the matrix-bound chondroitin sulfate chains. In vivo, a steric antiadhesive activity of chondroitin sulfate chains or keratan sulfate chains is also likely to require an immobilization of those chains to a rigid, immobile structure. Whether such a structure actually exists in brain is uncertain, although there are candidates, such as the roof plate of the spinal cord [90] and the optic chiasm [91]. However, repulsion by certain areas in vivo does not have to depend on steric antiadhesiveness, because it can be mediated by specific receptors induced by ephrins [92] or membrane-bound members of the semaphorin family [93]. Finally, it must be noted that as in all cell adhesion studies, the cultured cells confronted with a glycosaminoglycocalyx can escape into a matrix-free environment, the medium, an opportunity not available to cells in vivo. Interestingly, when HEK 293 cells secreting neurocan were aggregated in hanging drops and those aggregates were introduced into collagen gels, unlike aggregates of untransfected HEK 293 cells, the aggregated cells secreting neurocan dispersed [U. Talts and U. Rauch, unpublished observation].

The extracellular matrix of the brain

Structural peculiarities of the brain matrix

Neurocan is a component of the extracellular matrix of the brain. Since the brain is covered and protected by the skull, this tissue is not subjected to extensive tension and compression, and thus does not require a rigid connective tissue as do organs more exposed to such physical forces. Fibrillar collagens, hallmarks of many other connective tissues, are virtually absent from brain interstitial tissue, and cell-binding molecules like fibronectin, common to extracellular matrices of most other tissues, are only present during short periods of brain development [94]. However, since the brain retains its shape and integrity after removal of the skull, molecules mediating connective forces have to exist in this tissue as well. A lack of connective matrix components could be compensated by dozens of different cell-cell adhesion molecules of the Ig [48] and cadherin [89] superfamilies which would be able to contribute to the physical stability of the tissue. Ventricular dilatation observed in brains with mutated L1 molecules has been hypothesized as due to decreased mechanical tension by axons, dendrites, and glial processes [95]. Type IV collagen-, laminin-, and perlecan-containing endothelial and astroglial basement membranes which cover larger blood vessels of the brain in two layers [96] could support the physical stability of the brain. Therefore, other extracellular matrix molecules and their networks occupying the interstitial space might have to provide only a minor contribution to the physical stability of the tissue and might have acquired properties more important for the function of the brain.

Various extracellular matrix molecules occur exclusively, or in significant amounts, only in brain, among them neurocan and various other proteoglycans. The volume occupied by extracellular matrix in rat brain has been determined to be on average about 20% in adult, and about twice that in newborn animals [97]. Interestingly, the age-dependent changes correspond in large part to the decrease in the overall concentration of chondroitin sulfate in brain tissue [98]. Of the major proteoglycan species in brain, only neurocan and the large versican splice variants have a similar concentration profile, whereas the concentration of phosphacan and phosphacan-KS [99], brevican, and the smallest versican proteoglycan variant [68] increases. The content of neurocan appears to correlate with the volume of the extracellular space. In the molecular layer of the cerebellum, a compartment still containing significant amounts of neurocan in adult rat brain, the extracellular space occupies about 30% of the volume, considerably more than the average [97]. Similarly, an increase in the partial volume of the extracellular space has been observed after brain injuries, with consequent neuronal death and astrogliosis, a situation which is also characterized by reexpression of neurocan [82, 83].

One important function of the extracellular space is certainly the regulation of transport of diffusable molecules. In brain, these can be directional cues, growth and differentiation factors, neurotransmitters, or simple inorganic ions. In principle, the distance extracellular molecules are able to diffuse is inversely related to the ratio between extracellular and intracellular volume [100]. Therefore, the production of highly glycosylated and highly charged molecules like neurocan or other proteoglycans may be an efficient and energetically favorable way for cells to regulate the volume of the extracellular environment. Neurocan has, in addition, been shown to interact directly with several growth and differentiation factors, with fibroblast growth factor (FGF)-2, HB-GAM, and amphoterin, and could be involved in their sequestration, storage, or presentation. Neurocan also binds heparin, which can in turn interact with many other heparin-binding extracellular molecules, as for example netrins [101].

Possible roles of neurocan and interacting molecules

Neurocan can bind to various extracellular matrix components, and interact with several cell surface molecules. These interactions can modulate their activity, and especially inhibit homophilic interactions of neural cell adhesion molecules of the Ig superfamily and the cadherin family. For this latter activity, which is mediated by a GalNAcPTase, the same effect can be observed by using a mAb against this enzyme [64]. Thus, many of the effects observed after administration of antibodies or other artificial agents interfering with the function of molecules interacting with neurocan could mimic and obviously potentiate effects mediated by neurocan in vivo. Such observed effects include for example, reduced fasciculation of neurites growing out of cerebellar cortex explants [102], an arrest of most cerebellar granule cells in the external granular layer [103], interference with induction of long-term potentiation [104], amnesia for passive avoidance training in day-old chicks [105] and in adult rats [106], inhibition of neurite outgrowth on substrate-bound TAG-1 [107], and prevention of commisural growth cones from entering floor-plate explants [108]. A modest modulatory role for neurocan in many, but no absolutely essential role in any physiological process would be in line with the observation that neurocan knockout mice are viable, fertile, and exhibit no gross anatomical brain defects [X. Zhou and R. Faessler, unpublished observation]. Similar observations, at least with respect to viability and fertility, have been made with most of the mice lacking a molecule which interacts with neurocan, like tenascin-C [109, 110], tenascin-R [111], N-CAM [112], L1 [113], and TAG-1 [114].

However, there are indications that some of those molecules might be involved in the function of the brain rather than in determining its overall structure. TAG-1-deficient mice appeared normal by gross morphological analysis of the cerebellum, the spinal cord, and the hippocampus, but showed increased epileptogenicity and upregulation of adenosine A1 receptors in the hippocampus [114]. Abnormal behavior and neurotransmission were also reported from anatomically normal-appearing tenascin-C-deficient mice [115, 116]. The anatomy of all major brain areas and the formation and structure of myelin appeared normal in tenascin-R knockout mice [111], but they exhibited decreased axonal conduction velocities, reduced perisomatic inhibition, increased excitatory transmission, and impaired long-term potentiation [117]. Impaired long-term potentiation at the mossy fiber synapses has also been observed in N-CAM-deficient mice, although in this case, the functional deficits were accompanied by structural alterations, since fasciculation and laminar growth of mossy fibers were strongly disturbed in the mouse mutants [118]. Surprisingly, long-term potentiation was not affected in the hippocampus of mice lacking L1 [119], the molecule which when mutated causes varying degrees of brain malformation and mental retardation in humans [57].

Conclusion

Many observations indicate that the extracellular matrix of the brain with its special composition might be only peripherally involved in the development and preservation of the physical integrity of the brain, and mainly designed to serve brain function. This might be difficult to assess in such stupid animals like mice, but could therefore also be more challenging. Neurocan is a part of the matrix, mainly present during modeling and remodeling stages. It might contribute to the function of the matrix through its biophysical properties, its interactions with other molecules, and its susceptibility to proteolytic processing, since cleavage of matrix molecules at strategic positions could induce rapid changes in the consistency of the matrix, and facilitate endocytosis of its components, thereby increasing the ability of cells to remodel the matrix.

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