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Brevican-containing perineuronal nets of extracellular matrix in dissociated hippocampal primary cultures

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Perineuronal nets (PNN) are specialized extracellular matrix structures enwrapping CNS neurons, which are important regulators for neuronal and synaptic functions. Brevican, a chondroitin sulfate proteoglycan, is an integral component of PNN. Here, we have investigated the appearance of these structures in hippocampal primary cultures. The expression profile of brevican in mixed cultures resembles the in vivo pattern with a strong upregulation of all isoforms during the second and 3rd weeks in culture. Brevican is primarily synthesized by co-cultured glial fibrillary acidic protein (GFAP-)-positive astrocytes and co-assembles with its interaction partners in PNN-like structures on neuronal somata and neurites as identified by counterstaining with the PNN marker *Vicia villosa* lectin. Both excitatory and inhibitory synapses are embedded into PNN. Furthermore, axon initial segments are strongly covered by a dense brevican coat. Altogether, we show that mature primary cultures can form PNN, and that basic features of these extracellular matrix structures may be studied in vitro.

Keywords: Proteoglycan; Glia; Synapse; Primary culture; Axon initial segment; Perineuronal nets; Extracellular matrix

Introduction

The brain extracellular matrix (ECM) is a pericellular meshwork of glycoproteins, proteoglycans, and cell surface receptors, which mediate fundamental neural processes like cell migration, neurite outgrowth, synaptogenesis, injury-related plasticity, and synaptic stabilization. This matrix is formed by a not yet completely understood interplay of glial and neuronal components and clearly differs from the ECM of other tissues with respect to its biochemical properties, its molecular composition, and the complexity of ascribed functions (Rutka et al., 1988; Ruoslahti, 1996; Rauch, 1997). Thus, an astonishingly large variety of proteoglycans is found in the brain ECM.

Among the chondroitin sulfate proteoglycans (CSPGs), the family of lecticans plays a central role (Margolis and Margolis, 1994; Iozzo and Murdoch, 1996; Yamaguchi, 2000). This family comprises aggrecan and versican as widely expressed members and neurocan and brevican as neural-specific molecules. Lecticans are structurally characterized by a modular composition of conserved globular domains in the N- and C-termini and a central variable region. This domain composition reflects a functional segregation of several binding interfaces like the N-terminal hyaluronic acid (HA)-binding region and the C-terminal cell-binding and ECM-interconnecting motifs. Lecticans can build up complex carbohydrate–protein networks with HA, also termed hyaluronan, which is the central organizing polysaccharide of the neural ECM, thus contributing to the organization of the extracellular space.

Complexed with glycoproteins, e.g., tenascins, some of the lecticans create net-like specializations of the brain ECM, the so-called perineuronal nets (PNN). PNN are formed late in development, and they selectively enwrap large neurons to create a suitable polyanionic microenvironment (for review, see Celio and Blumcke, 1994; Murakami and Ohtsuka, 2003). Although these extracellular meshwork structures are known for more than a century due to the pioneering work of Cajal and Golgi (summarized in Celio et al., 1998), their functional significance has long been under debate, since the knowledge about their constituents is still sparse. Nowadays, PNN are discussed as fast local buffers for strong variations in the extracellular cation concentration (Härtig et al., 1999) and as neuroprotective shield against oxidative stress (Morawski et al., 2004). Moreover, the hypothesis has been proposed that they might act in the context of stabilization, electrical insulation, and growth factor supply of synapses, hence contributing to the mature neurotransmission properties of the CNS (summarized in Brückner et al., 1993; Celio et al., 1998; Murakami and Ohtsuka, 2003).

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Brevican is one of the most prominent constituents of mature rodent brain ECM (reviewed in Gary et al., 1998, Yamaguchi, 2000). In contrast to its sister molecule neurocan (reviewed in Rauch et al., 2001), which is characteristic for immature brain ECM, brevican expression is strongly upregulated during postnatal development, when bulk synaptogenesis occurs. Brevican is a conditional chondroitin sulfate proteoglycan with a tripartite structure. The C-terminal lectin domain has been shown to interact specifically with the extracellular glycoprotein tenascin R (TN-R; Aspberg et al., 1997; Brückner et al., 2000) and with sulfated glycolipids at the cell surface (Miura et al., 1999; Miura et al., 2001). The central region contains matrix metalloprotease cleavage sites. Specific proteolytic cleavage leads to the separation of the N-terminal HA-binding region from the C-terminal cell surface- and protein-interacting part of the molecule and may have implications for the integrity and biophysical features of brain ECM.

In contrast to the other lecticans (i.e., neurocan, versican, and aggrecan), brevican exists in different splice isoforms generated by alternative transcript processing in the 3′ region (Seidenbecher et al., 1995; Rauch et al., 1997). The more abundant 3.6-kb mRNA encodes the prominent secreted brevican isoform, whereas the less abundant 3.3-kb transcript gives rise to the smaller variant, which lacks the C-terminal globular domains and instead harbors an attachment sequence for a glycosylphosphatidyl inositol (GPI) anchor, which ties this isoform to the cell membrane (Seidenbecher et al., 1995, 2002) and makes it a cell surface HA receptor.

Brevican was shown to be a structural constituent of PNN (Hagihara et al., 1999; Brückner et al., 2000). Within PNN, it co-localizes with the brain-specific link protein Bral2 (Bekku et al., 2003) and its interaction partner TN-R (Hagihara et al., 1999), and in mice lacking a functional TN-R gene, the PNN appearance is changed with less brevican found in these nets (Brückner et al., 2000). Accordingly, PNN in brains of brevican-deficient mice display a less structured and more diffuse appearance (Brakebusch et al., 2002). Brevican staining is generally widely distributed in the rodent brain but shows perisynaptic deposits of immunoreactivity at the ultrastructural level (Seidenbecher et al., 1997; Hagihara et al., 1999). Electrophysiological studies on brevican-deficient mice revealed a phenotype of significantly impaired synaptic plasticity (Brakebusch et al., 2002).

Synapses are known to be rich in several carbohydrates bound to glycoproteins, most of these functioning as receptors for neurotransmitters, neurotrophins or ECM molecules. However, much less is known about secreted extracellular proteoglycans or glycoproteins found at or near synapses. Only recently, for a couple of ECM constituents, a synaptic localization and function in the context of LTP could be documented (reviewed in Dityatev and Schachner, 2003). Besides brevican and neurocan, these include their interaction partners TN-R and TN-C, respectively, as well as reelin and laminin, both of which can activate identified intracellular signaling cascades. For the proteoglycans, however, the mode of synaptic action is still enigmatic, and it is a challenging task to unravel these mechanisms. Furthermore, it is not completely understood, how single components are integrated into PNN, how these networks are anchored at neuronal surfaces, and how these relatively stable structures relate to plastic brain properties.

During the past decade, much insight into molecular components affecting synaptogenesis, synaptic structure, and function has been obtained from studying cultured hippocampal primary neurons (for review, see Verderio et al., 1999). Brevican is detected in these cultures decorating the surface of neurons, and the immunoreactivity is diminished after hyaluronidase or chondroitinase ABC treatment (Seidenbecher et al., 2002). In the present paper, we address the question whether proteoglycan-containing PNN structures, thus far thought to be a hallmark of intact mature neural tissue, can be formed in dissociated neural cultures. We analyze the cellular source of brevican in mixed neuron-glia co-cultures, the temporal and spatial appearance of brevican-containing PNN and the relation of these nets to synaptic contact sites.

Results

Brevican expression in culture parallels the expression profile in the brain

As shown previously, brevican is a component of the mature brain ECM, which is synthesized relatively late during ontogenetic development, coinciding with synaptogenesis and final wiring in the brain (Seidenbecher et al., 1995, 1998). As documented in Fig. 1, brevican expression in primary cultures follows a very similar time course. The immunoreactivity is barely detectable on young MAP2-positive neurons of 1-week-old cultures but becomes more intense after the 2nd week in vitro and is very prominent on 21-day-old neurons, which display a mature morphology (Figs. 1A–F). Since the antibody used for immunostaining cannot discriminate between the different brevican isoforms, we performed an immunoblot analysis to investigate their presence in the cultures (Fig. 1G). The blot clearly shows that brevican immunoreactivities from hippocampal cultures and from brain extracts display the same migration behavior with detectable protein bands between 145 and 120 kDa. Moreover, the attachment of CS side chains sensitive to Chondroitinase ABC treatment becomes stronger during the 2nd week of cultivation. The relative amount of the 80-kDa fragment, which is generated by proteolytic cleavage (Seidenbecher et al., 1995), increases during this time period. The latter observation indicates that the proteolytic activity necessary to perform the partial cleavage is present under in vitro culture conditions as well. In order to estimate the relative increase of brevican during culture maturation, the optical densities were measured and normalized relative to actin. The total brevican immunoreactivity increases approximately 1.5- to 2-fold within the second and 3rd week in culture (Fig. 1H). This finding is in good agreement with the brevican expression profile in the rodent CNS. These data show that during maturation of the primary cultures, brevican is strongly upregulated, partially cleaved and modified with sulfated carbohydrate side chains. This closely resembles the expression and modification profile of the in vivo expressed proteoglycan (Seidenbecher et al., 1995).

Interestingly, the culture media contain almost exclusively full-length brevican, which is only sparsely modified with CS side chains (Fig. 1J). This finding indicates that CS-modified brevican is preferentially bound by the cells, while the core protein is retained in the medium.

Primary cultures express secreted and GPI-brevican at a similar ratio than intact rat brain

In the brain, brevican is known to be expressed as two different splice isoforms (Seidenbecher et al., 1995). Therefore, we analyzed