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Developmental patterns of proteoglycan-containing extracellular matrix in perineuronal nets and neuropil of the postnatal rat brain

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Abstract. The extracellular matrix is involved in various morphogenetic processes which are accompanied by changes in its physicochemical properties and spatial organization. In the adult brain it contributes to cellular communication and the regulation of neuronal activity. The present study deals with the postnatal appearance and transformation into adult distribution patterns of extracellular matrix components related to chondroitin-sulphate proteoglycans (CSPGs) in the rat brain. The differential accumulation of these components in neuropil and in perineuronal nets (PNs) enriched in certain regions was examined in 0-, 7-, 14-, 21- and 35-day-old rats and adult animals using the *N*-acetylgalactosamine-binding *Wisteria floribunda* agglutinin (WFA) and immunocytochemical detection of CSPGs. The lectin stained the olfactory-bulb glomerular layer and layer Ia of piriform and entorhinal cortex already in newborn animals. On postnatal day 7 diffuse neuropil staining was additionally found in certain subcortical nuclei and in deep neocortical layers. The first sharply contoured PNs were detected at this age in the brain stem, indicating the more advanced maturation of matrix components in subcortical regions. CSPG immunoreactivity yielded staining patterns largely identical to WFA-binding patterns but appeared only between postnatal day 14 and 21. The adult-like stage was revealed with both methods between 21 and 35 days after birth. The results provide further evidence that the accumulation of certain CSPGs in the extracellular space is spatiotemporally related to distinct patterns of neuronal activity at the regional and cellular level.

Key words: Ontogenesis – Perineuronal nets – Extracellular matrix – Proteoglycans – Brain – Rat (Wistar)

Introduction

In the adult mammalian brain various authors have described lattice-like coatings around certain types of neurons distributed in a high number of cortical and subcortical brain regions (Brückner et al. 1993; reviewed in Celio and Blümcke 1994). These coatings, which are perforated by synaptic boutons, have a net-like appearance and are therefore called perineuronal nets (PNs) (Brauer et al. 1982, 1984). In a number of studies dealing with the structure and chemical nature of PNs it was shown that they represent accumulations of extracellular matrix composed of large aggregating chondroitin-sulphate proteoglycans (CSPGs) associated with hyaluronan (DeYoe et al. 1990; Bignami et al. 1992; Brauer et al. 1993; Brückner et al. 1993; Bertolotto et al. 1996). These components have been cytochemically detected by the use of specific antibodies, lectins and basic dyes (Nakagawa et al. 1987; Bignami et al. 1992; Girard et al. 1992; Härtig et al. 1992; Brückner et al. 1993, 1996a; Schweizer at al. 1993; Celio and Blümcke 1994; Yasuhara et al. 1994; Bertolotto et al. 1996). The function of the CSPGs in the PNs has been the subject of recent investigations and is still controversely discussed. Several possible functions have been suggested for these structures, such as stabilization of synaptic contacts, involvement in neuronal information processing and protection of neurons against toxic influences (Hockfield et al. 1990; Brückner et al. 1993; Celio and Blümcke 1994). In addition to the more or less clearly contoured PNs, a more diffuse distribution of CSPGs was described in the neuropil in certain layers of the cortex or regions in the subcortical grey matter (DeYoe et al. 1990; Brückner et al. 1994; Seeger et al. 1994; Bertolotto et al. 1996). These components characterize the microenvironment of nonmyelinated, preterminal axonal processes (Brückner et al. 1996b).

This paper is dedicated to Professor Andreas Oksche on the occasion of his 70th birthday, in recognition of outstanding contributions to neuroscience and to the internationalisation of science.

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The present study deals with the postnatal appearance of PNs and the distribution of extracellular matrix components in the neuropil and their course of maturation throughout the rat brain, which has not been previously systematically studied. The purpose of this study is to contribute to the view of the extracellular matrix as an integral part of the spatiotemporal pattern of neuronal maturation. The markers used for the demonstration of PNs are the plant lectin *Wisteria floribunda* agglutinin (WFA) and antibodies against CSPGs both of which have been proven to be sensitive tools for staining perineuronal nets in adult animals (Brückner et al. 1994; Härtig et al. 1994). From the various brain regions in which those extracellular matrix components appear first in ontogenesis, four regions were selected for a more detailed analysis on the basis of the different temporal pattern of appearance of extracellular matrix zones and their very distinct developmental characteristics.

Materials and methods

Animals and tissue processing

Wistar rats on postnatal days (PD) 0, 7, 14, 21, 35 and 2-month-old animals were transcardially perfused under deep Velonarcon anaesthesia (ketamine hydrochloride; Berlin-Chemie) with saline, followed by 150 ml of 4% paraformaldehyde in 0.1 M sodium-phosphate buffer, pH 7.4 (PB). The brains were thereafter frozen in *n*-hexane at –70°C for 30 s. Thirty-micron sections were treated with 0.6% H_2O_2 in 0.1 M TRIS-buffered saline, pH 7.4 (TBS), for 30 min to block endogenous peroxidase activity and rinsed with TBS.

Detection of N-acetylgalactosamine by WFA

The detection of *N*-acetylgalactosamine was performed with reduced biotinylated WFA (Sigma, Deisenhofen) used at a concentration of 1.0 µg/ml TBS–BSA (TBS containing 2% bovine serum albumin; Serva, Heidelberg). Sections were incubated overnight at 4°C with WFA, processed with a streptavidin–biotin–peroxidase complex (ABC) and revealed with diaminobenzidine (Sigma) enhanced by nickel ammonium sulphate (DAB/Ni) as chromogen (Härtig et al. 1992). After sufficient rinsing with buffer and distilled water, sections were mounted in Entellan (Merck, Darmstadt) and covered with a coverslip. In control sections WFA was omitted from the incubation or used after preincubation with 200 mM *N*-acetylgalactosamine.

Detection of CSPGs

The protocol applied mainly followed a recently described procedure (Härtig et al. 1994). Briefly, unspecific binding sites for immunoreagents were blocked with 5% normal goat serum in TBS (TBS–NGS). Subsequently, sections were processed with a rabbit antiserum raised against bovine nasal cartilage CSPG (anti-CSPG; Chemicon, Temecula, CA; Bertolotto et al. 1986) at a dilution of 1:6000 in TBS–NGS overnight at 4°C followed by incubation with biotinylated goat-anti-rabbit IgG (1 µg/ml TBS–BSA; Dianova, Hamburg) and ABC for 1 h each. The staining of sections was carried out with DAB/Ni. In control experiments either the primary antibody or the primary and secondary antibodies were omitted.

Series of sections of every brain were preincubated with chondroitinase ABC (EC 4.2.2.4) from *Proteus vulgaris*. Chondroitinase ABC was applied at a concentration of 0.5 U/ml 40 mM TRIS-HCl buffer, pH 8.0, containing 40 mM sodium acetate and 0.1% BSA for 90 min at 37°C. Thereafter the sections were treated with WFA or anti-CSPG which were visualized as described above. The individual brain regions were identified according to the stereotaxic atlas of Paxinos and Watson (1986).

Results

General remarks

The first appearance of WFA-stained components can be detected between the day of birth and PD 14, depending on the region. The adult-like staining was attained between PD 21 and PD 35 (Figs. 1–3). The data obtained for different cortical and subcortical regions are summarized in Table 1. The maturation of matrix components accumulating in PNs or the neuropil of some selected brain regions is described in detail below, taking into account the developmental stages PD 0, PD 7, PD 14, PD 21, PD 35 and 2-month-old rats.

The deposition of extracellular matrix components was not transient in nature. It increased its intensity continuously and developed its structural features up to the adult-like stage. In newborn animals all intensely stained neuropil zones were related to the olfactory system. The staining of PNs became visible in subcortical regions prior to those located in the cerebral cortex.

The CSPG-immunoreactivity staining pattern was essentially the same as the WFA-binding pattern but appeared to be delayed between PD 14 and 21. Only some regions such as the glomerular, external and internal plexiform layer of the olfactory bulb, layer Ia of the piriform cortex and the red nucleus showed a weak staining at PD 14. The neuropil and PNs could be demonstrated in all brain regions listed in Table 1 at PD 21 by CSPG immunoreactivity in a similar way as visualized by WFA-binding. As an example, Fig. 3F demonstrates CSPG-immunoreactivity in the red nucleus.

Treatment of sections with chondroitinase ABC had no influence on the staining pattern obtained with anti-CSPG, but prevented WFA-binding, which indicated the attachment of WFA to glycosaminoglycan side chains of CSPGs also in juvenile brains. This conclusion was already drawn from previous comparative labelling studies in adult animals (Härtig et al. 1994; Köppe et al., in press).

Olfactory bulb

The olfactory bulb of newborn rats showed a rather continuously WFA-labelled glomerular layer (Fig. 1A). In contrast, at PD 7 the individual glomeruli appeared clearly outlined and more intensely stained (Fig. 1B). The external and internal plexiform layers were additionally diffusely labelled. Whereas the glomeruli were most heavily labelled, the external plexiform layer was weakly stained and the internal plexiform layer exhibited moderate staining (Fig. 1B). The intensity of label was found to be increased at PD 14 (Fig. 1C) and reached the adult stage intensity (Fig. 1D) at PD 21. The two plexiform layers and the glomeruli were not recognizable be-

Table 1. Postnatal maturation of extracellular matrix revealed by *W. floribunda* agglutinin in neuropil and perineuronal nets (PNs) of rat brain. The first appearance of stained components is compared with the detection of an adult-like pattern as indicated by clearly contoured PNs

	First detection of neuropil (PD)	First detection of of immature PNs (PD)	Detection of adult-like PNs (PD)
Cerebral cortex and hippocampus			
Entorhinal cortex	0	14	35
Fields CA 1–3 of Ammon's horn	7[a]	14	35
Olfactory bulb	0	–	21
Occipital cortex, area 1	7	14	35
Occipital cortex, area 2	7	14	35
Parietal cortex, area 1	7	7	35
Parietal cortex, area 2	7	14	35
Piriform cortex	0	14	35
Retrosplenial cortex, granular and agranular	7[a]	14	35
Temporal cortex, area 1	7	14	35
Temporal cortex, area 2, 3	7	14	35
Subcortical areas			
Cerebellar nuclei	7	7[a]	21
Deep gray layer of superior colliculus	14[a]	14	21
Deep mesencephalic nucleus	14	14	21
Dorsolateral septal nucleus	14	14	21
External cortex of inferior colliculus	7	7	21
Gigantocellular reticular nucleus	–	0	21
Lateral vestibular nucleus	14	14	21
Medial septum and diagonal band nuclei	7	7	21
Motor trigeminal nucleus	14	14	21
Nucleus of the brachium of inferior colliculus	7	7	21
Oculomotor nucleus	7[a]	14	21
Red nucleus	7	7	21
Reticular thalamic nucleus	7[a]	14	21
Substantia nigra, reticular part	14	14	21
Superior olive	7	14	21
Trapezoid body, medial part	7	7	21
Ventral cochlear nucleus	14	14	21
Zona incerta	7	14	21

[a] Very weakly stained compared to the adult labelling

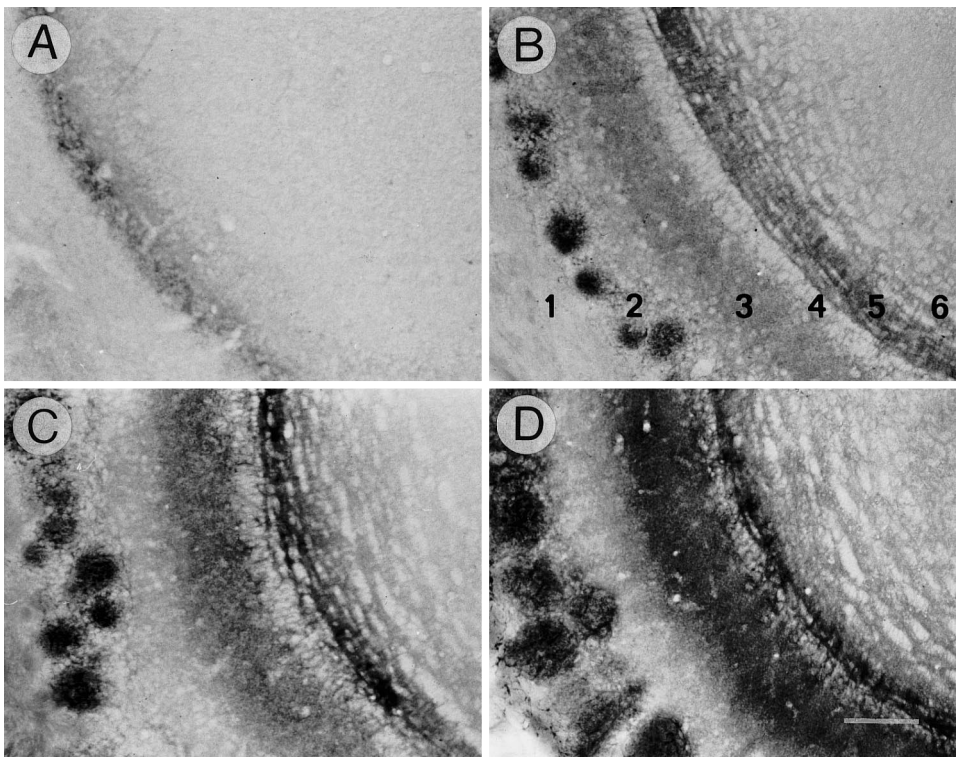

Fig. 1A–D. Developmental pattern of *W. floribunda* agglutinin-positive structures in the olfactory bulb. A diffuse staining of the glomerular layer is observed in newborn rats (**A**) and the characteristic adult-like pattern of neuropil staining is evident already on the PD 7 (**B**). The staining intensity increases on PD 14 (**C**) and in 2-month-old rats (**D**). *1*, Olfactory nerve layer; *2*, glomerular layer; *3*, external plexiform layer; *4*, mitral cell layer; *5*, internal plexiform layer; *6*, internal granular layer. ×100. *Bar:* 30 μm

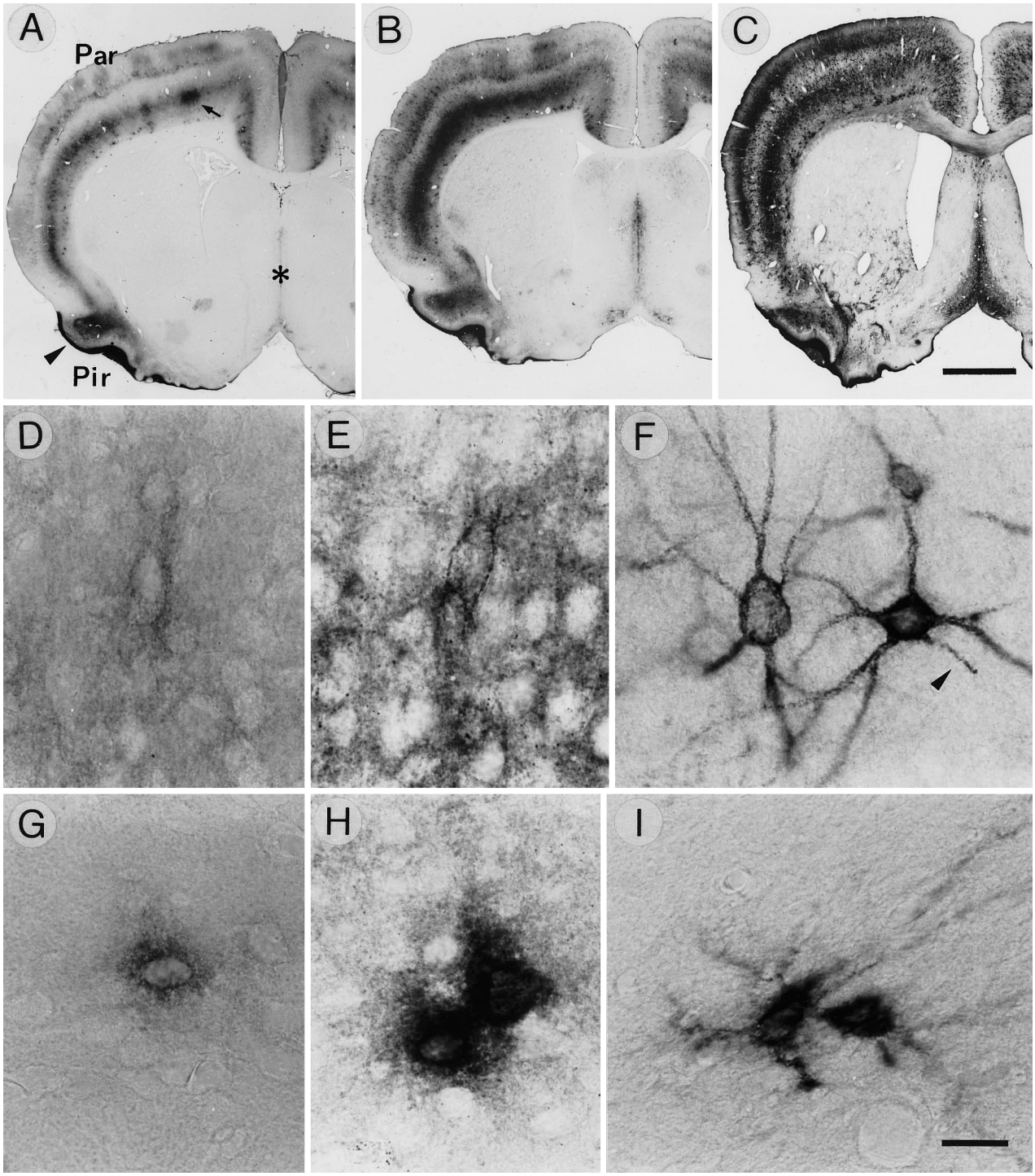

Fig. 2. Labelling of extracellular matrix components by *W. floribunda* agglutinin in the rat forebrain on PD 7 (**A, D, G**), PD 14 (**B, E, H**) and at 2 months (**C, F, I**). At low magnification (**A–C**) characteristic distribution patterns are revealed in the cerebral cortex. On PD 7, layer Ia (*arrowhead*) in the piriform cortex (*Pir*) appears intensely labelled. Diffuse staining occurs in the parietal cortex (*Par*) yielding a two-tiered pattern with intensely stained patches such as at the presumptive forelimb area (*arrow*). The first appearance of lectin binding in the basal forebrain can be observed in the medial septum/diagonal band complex (*asterisk*). Faintly labelled PNs ensheathing cell bodies can first be detected in layers IV (**D**) and VI (**G**) of the parietal cortex at the end of the first week. In the upper layers the nets can hardly be distinguished from the surrounding neuropil at the end of the second week (**E**) but they are clearly contoured also on proximal parts of dendrites and the initial segment of axons (*arrowhead*) in the adult stage (**F**). Some neurons in layer VIb (**G–I**) have the characteristic "fuzzy" appearance already on PD 7. **A–C**×7.2; *bar:* 1.5 mm; **D–I**×630; *bar:* 5 μm

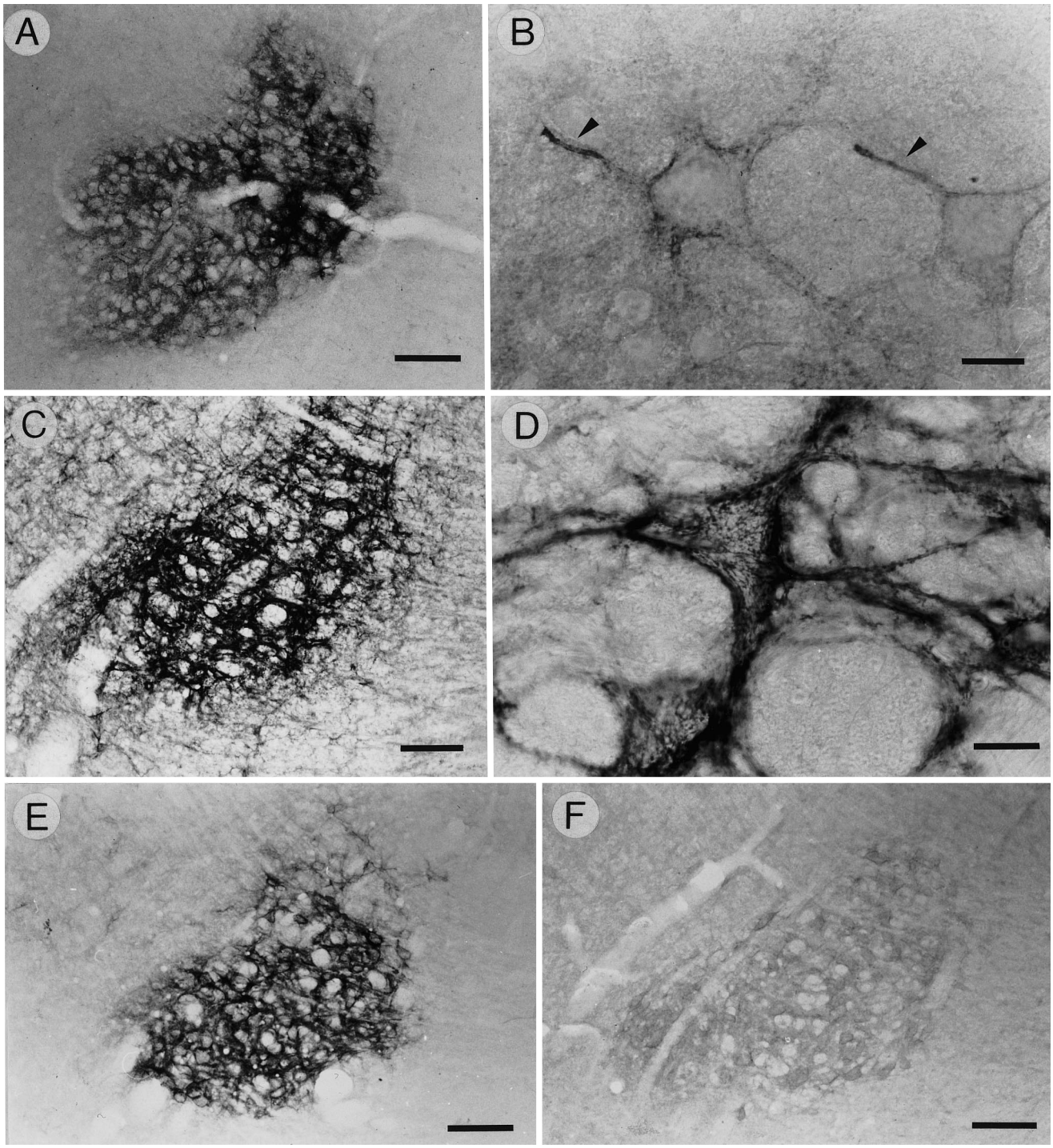

Fig. 3. Demonstration of PNs in the red nucleus from PD 7 rats (**A**, **B**), PD 14 rats (**E, F**) and from adult rats (**C**, **D**). Some magnocellular neurons have lattice-like sheaths composed of lectin-stained matrix components already on PD 7 (**B**). *Arrowheads* indicate initial segments of axons. The staining patterns obtained with *W. floribunda* agglutinin (**E**) and a rabbit antiserum directed against CSPGs (**F**) in 14-day-old rats show that the red nucleus and its individual PNs are revealed less selectively by the immunoreaction. **A**, **C, E, F**×100; *bar*: 30 µm; **B**, **D**×630; *bar*: 5 µm

fore PD 14 in bulb sections incubated with anti-CSPG. At PD 21 the CSPG immunoreaction was the same as that of the adult.

Piriform cortex

Like the glomerular layer in the olfactory bulb, the neuropil of the piriform cortical layers Ia and III was already stained at PD 0. The neuropil of layer Ia appeared considerably darker than that of layer III. At the end of

the following week the accumulation of WFA-positive components was increased, resulting in an intensely stained neuropil (Fig 2A). In layer Ia the staining intensity of the adult stage was reached at PD 14 (Fig. 2B). In layer III of 14-day-old rats fluffy perisomatic sheaths around some neurons appeared. Three weeks after birth the number of stained perineuronal net-like structures was found to be increased, and axon initial segments as well as proximal parts of dendrites were often clearly outlined. In the adult the PNs in layer III appeared more numerous than at PD 21. At PD 35 the PNs were distinctly labelled and more distal parts of dendrites were also ensheathed. The distribution and appearance of PNs as well as the labelling of the neuropil was the same as that of the adult (Fig. 2C).

Fourteen days after birth the neuropil of layer Ia was faintly labelled with anti-CSPG. At PD 21, the anti-CSPG staining pattern of the piriform cortex was similar to the WFA staining pattern. Its mature anti-CSPG staining pattern was also achieved at PD 35.

Parietal cortex

At PD 0 only a faintly WFA-stained band in the deeper part of the cortex was visible. Seven days later a diffuse and more intensely stained two-tiered pattern was observed which may be attributed to layer IV and the upper layer VI; the staining was stronger in the latter layer (Fig. 2A). Area-specific differences in staining intensity became evident at this stage. For instance, in the region which presumably represents the forelimb area, the upper layer VI was more strongly labelled than that of other areas such as the frontal cortex which showed additional staining in layers II/III (Fig. 2A). In the upper layers neuronal surrounds resembling the earliest stages of PNs were recognized on PD 7 (Fig. 2D). Perisomatic accumulations around some neurons of layer VIb were intensely labelled (Fig. 2G). At PD 14 the staining intensity was considerably increased but the overall pattern remained similar to that of PD 7. Perineuronal-net-like sheaths were distributed throughout all layers except layer I (Fig. 2B,E,H). A further increase in the staining intensity occurred between PD 14 and 21. Many neurons were surrounded by clearly contoured perisomatic coatings, which also ensheathed initial segments of axons and proximal parts of dendrites. At PD 35 the staining resembled the adult-like pattern (Fig. 2C,F,I). CSPG-immunopositive matrix components were first detected at PD 21. The structural appearance and distribution of anti-CSPG-labelled PNs was similar to that of PNs labelled by WFA.

Red nucleus

In the red nucleus of newborn rats no lectin binding was observed. However, faintly stained PNs occurred in the tegmental reticular formation. At PD 7 heavily stained PNs ensheathing the neurons of the red nucleus were detected. These did not have the shadow-like appearance as in the cortex, but were rather sharply demarcated. The PNs of the parvocellular part of the red nucleus were more strongly labelled than those of the magnocellular part (Fig. 3A). PNs of the latter region exhibited a similar staining intensity as the neuropil in the parvocellular region. Initial segments of axons were distinctly recognizable (Fig. 3B). At the end of the second postnatal week the staining intensity was further increased, but it did not reach that of the adult stage. The PNs of 21-day-old rats resembled those in the adult brain in which the main portion of the neurons was surrounded by clearly contoured WFA-positive lattice-like coatings (Fig. 3C,D). In contrast to the heavy WFA-staining at PD 14 (Fig. 3E) the CSPG immunoreactivity of the red nucleus was relatively low at this stage (Fig. 3F). However, the structural development of PNs revealed by both methods was similar.

Discussion

The results of this study indicate that the accumulation of the extracellular CSPGs in the neuropil and in PNs, as revealed by WFA-binding and CSPG immunoreactivity, is a continuous rather than a transient postnatal process. Its spatiotemporal pattern appears to be related to the functional maturation of individual neuronal systems, which is also evident in other developmental processes such as neurogenesis, synaptogenesis or myelination of brain regions (Jacobson 1963; Aghajanian and Bloom 1967). The formation of locally enlarged extracellular matrix zones (Brückner et al. 1996b) is accompanied by a general decrease in the extracellular volume fraction during postnatal development (van Harreveld 1972; Lehmenkühler et al. 1993).

Like in the adult, in the developing rat brain the *N*-acetylgalactosamine-binding lectin WFA and CSPG immunoreactivity exhibit largely identical distribution patterns (Härtig et al. 1994; Köppe et al., in press), indicating that both labels reveal components of extracellular-matrix proteoglycans in closely related positions. This correlation is further supported by the fact that WFA-labelling is abolished after chondroitinase treatment as shown in this study and in previous studies (Köppe et al., in press).

WFA-binding compared with spatiotemporal patterns of different extracellular matrix components during development

According to their various functions in development the various extracellular matrix components are expressed in characteristic temporal patterns. The postnatal appearance of WFA-binding in the rat has certain similarities to the late development of large aggregating CSPGs such as the aggrecan-related proteoglycan Cat-301 in the feline visual system (Sur et al. 1988; Hockfield et al. 1990). Versican, a member of the same proteoglycan family, was first observed in the perineuronal coats on day 21 in the rat cerebral cortex (Bignami et al. 1993).

In contrast, other CSPGs, such as neurocan and phosphacan, are transiently expressed during early periods of brain development (see e.g. Margolis and Margolis 1989; Rauch et al. 1991; Oohira et al. 1994 a, b; Engel et al. 1996; Meyer-Puttlitz et al. 1996). Hyaluronic acid, involved in cell proliferation and migratory processes, is most highly concentrated in the embryonic brain and declines postnatally, with a significant drop after PD 10 (Delpech et al. 1987; Margolis and Margolis 1989). However, hyaluronic acid remains as a ubiquitous component of the extracellular matrix in PNs and neuropil in association with proteoglycans produced mainly postnatally (Delpech et al. 1989; Bignami and Asher 1992).

Extracellular chondroitin-sulphate proteoglycans related to functional maturation of brain areas and individual neurons

A comparison of the sequence of maturation of WFA-stained matrix components with the time schedule of neurogenesis in the rat brain (Bayer and Altman 1995) suggests that the former does not completely mimic the temporal pattern of the preceding neurogenetic period. Rather, its temporal maturation sequence appears to be related to the increasing behavioural complexity of the developing animals. An example is the olfactory system which is functionally active at an immature level already in newborn rats when olfactory-bulb interneurons are still proliferating (Bayer 1983; reviewed in Brunjes and Frazier 1986). At this age the WFA-positive layer Ia in the piriform and entorhinal cortex related to olfactory bulb output projections (Wouterlood and Härtig 1995) represents the most intensely WFA-stained structure in the brain. The WFA staining of the neuropil in the olfactory bulb glomerular layer accompanies the transformation of olfactory afferents into glomeruli which is temporally coincident with the transient expression of cytotactin/tenascin and certain CSPG species associated with astroglial cell processes (Gonzalez et al. 1993). In contrast, WFA-stained PNs in the regions of the olfactory bulb projection reach their mature appearance only during the third postnatal week, indicating their involvement in more complex functions performed in the adult olfactory system. In the neocortex the strong WFA-binding of the neuropil in deeper layers and the perineuronal labelling in layer VIb at the end of the first postnatal week may be considered as a concomitant of the more progressed maturation of deep cortical layers. On the other hand, these distribution patterns of the extracellular matrix CSPGs also suggest a spatial relation to input areas of functionally active thalamocortical fibres which arborize extensively in deeper layers before reaching layer IV in subsequent stages (Agmon et al. 1993). The first indication of the structural and functional segregation of the neocortex into individual areas as revealed by WFA-labelling in adult rats (Brückner et al. 1994; Seeger et al. 1994) is a patchy neuropil staining during the first postnatal week. However, these patterns are obviously complementary to those described for extracellular matrix components related to astroglial barriers or boundaries between neuronal units (Snow et al. 1990). As shown in the developing barrel field of the parietal cortex, a variety of these matrix molecules such as neurocan (Oohira et al. 1994b; Watanabe et al. 1995), ABAKAN, which is a keratan-sulphate proteoglycan (Geisert and Bidanset 1993), cytotactin and cytotactin-binding proteoglycan (Jhaveri et al. 1991), or tenascin (Steindler et al. 1989; Sheppard et al. 1991) either spare the barrel centres already in early development or are downregulated during the postnatal period (Faissner and Schachner 1995; Faissner and Steindler 1995).

Since the main periods of synaptogenesis as described for some cortical areas (Aghajanian and Bloom 1967; Blue and Parnavelas 1983) roughly correspond with the time course of perineuronal accumulation of CSPGs, a role in the termination of synaptic plasticity related to synaptic stabilization, was suggested (Sur et al. 1988; Hockfield et al. 1990; Schweizer et al. 1993). This notion was experimentally supported by findings in the monocularly deprived kitten in which a reduced number of PNs in the visual system has been demonstrated (Sur et al. 1988; Guimarães et al. 1990; Kind et al. 1995). Many other data show that the maturation of PNs proceeds in a temporal pattern which corresponds to the progression of myelination (Jacobson 1963). Thus, the accumulation of perineuronal CSPGs may be viewed in the context of the maturation of the high conduction velocity of neurons.

As suggested by studies from adult animals of different mammalian species, in many brain regions the PNs are associated with fast-spiking types of neurons (Kawaguchi et al. 1987) and streams of rapid information processing (DeYoe et al. 1990; Guimarães et al. 1990). In the cortex and several subcortical regions a major population of these neurons contains the calcium-binding protein parvalbumin (see e.g. Kosaka and Heizmann 1989; Kosaka et al. 1992; Härtig et al. 1994). Regarding their developmental pattern in the rat, parvalbumin immunoreactivity develops 3 weeks to 1 week earlier than the WFA labelling demonstrated in our study (Solbach and Celio 1991). As these authors suggested, parvalbumin appears when the cells start to become active. The appearance of WFA-labelled extracellular material, in contrast, may be interpreted as a sign of progressively maturating neuronal activity. This conclusion is supported by the fact that the voltage-gated K^+-channel subunit Kv3.1b in parvalbumin-immunoreactive neurons of the hippocampus (Weiser et al. 1995) reaches its developmental maximum at PD 14 (Du et al. 1996) when PNs are first detectable in this region.

The results of the present study suggest that the first appearance and the changing patterns of WFA-labelled extracellular matrix CSPGs are related to the increasing neuronal activity with respect to its region-dependent developmental patterns. The postnatal formation of PNs proceeds in concert with main periods of myelination and synaptogenesis and the development of certain voltage-gated ion channels.

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