INTRODUCTION

Studies on behavioral recovery following damage to the brain implicate the significance of both precise developmental age of the experimental animals and unilaterality of the lesion (Kolb, 1996). Restricted unilateral cortical lesions of the neonatal kind may allow a better behavioral outcome than do similar lesions in adulthood (Kolb and Gibb, 1991; Pekovic et al., 2006). Although our knowledge of cellular and molecular mechanisms required to stimulate repair mechanisms and permit regeneration in the CNS is on the increase, it is not yet sufficient to give us a satisfactory answer.

Also, it is well known that CNS axons do not regenerate as readily as peripheral axons following traumatic brain injury, and that adult neurites fail to regenerate after CNS damage (Richardson et al., 1980; Aquayo et al., 1981; Rossi et al., 1995; Pekovic et al., 2006; Galtrey and Fawcett, 2007) because CNS axons have an intrinsically reduced capacity for growth in correlation with embryonic or early postnatal axons. The observed differences of regenerative capability depend on local environmental factors (Bray et al., 1981; Stoll et al., 1989) and/or the maturational stage of growing axons (Kapfhammer and Schwab, 1994). Generally, axonal outgrowth of axotomized adult neurons is the result of interplay between intrinsic changes in the genetic program of neurons (Schwab et al., 1993; Kapfhammer and Schwab, 1994; Caroni, 1997) and changes in permissiveness of the local environment (Schwab et al., 1993; Schwab, 1996; Schwab and Brosamle, 1997), implying a balance between molecules that promote growth and ones that inhibit it. Among molecules
which have both growth-promoting and growth-inhibiting activities is the heterogeneous class of molecules designated as proteoglycans (PGs) (Snow et al., 1990; McKeon et al., 1991; Letourneau et al., 1994; Faissner et al., 1994; Davies et al., 1997, 1999; Fawcett and Asher, 1999; Asher et al., 2001, 2002). The extreme variability of PG molecules (composed of different molecular masses and a protein core with a varying number of sulfate residues per disaccharide units or glycosaminoglycans - GAGs) might explain diversity of the functions of PGs and their apparent conflicting effects on neurite outgrowth during development and regeneration of the nervous system (Wight et al., 1992; Bovolenta and Fernaud-Espinosa, 2000). Chondroitin sulfate PGs (CSPGs) are molecules known to contribute to scarring at the site of CNS injury and represent a family of putative inhibitory ECM molecules that may limit axonal regeneration after the injury (Galtréy and Fawcett, 2007). To be specific, they are expressed for the most part in areas that serve as a barrier to growing axons during development and regeneration (Snow et al., 1990; Dou and Levine, 1994; Friedlander et al., 1994; Asher et al., 2002; Jones et al., 2002; Rhodes et al., 2003; Rhodes and Fawcett, 2004). Various lines of evidence suggest that these molecules are upregulated after CNS injury. Thus, increased expression of CSPGs in the damaged CNS may limit the capacity for axon regeneration (McKeon et al., 1991; Pindzola et al., 1993; Davies et al., 1997, 1999, Gilbert et al., 2005).

In previous papers (Vasic et al., 1998; Pekovic et al., 2005, 2006), we showed that after ablation of the sensorimotor cortex in neonatal rats, reactive astrocytes were concentrated around the wound, but did not make a gliotic scar at the lesion site until the 30th day post-lesion. In addition, functional recovery was better than in adult animals, where intensive reactive astrogliosis was followed by the appearance of a glial scar two weeks after the ablation. Since CSPGs are present in areas of reactive gliosis (McKeon et al., 1999) we hypothesized that lesions provoke different patterns of expression of CSPGs after neonatal vs. adult lesions. In this study, we therefore examined the expression profile of two structurally related GAG epitopes, chondroitin-4-sulfate PG (CS-4PG) and chondroitin-6-sulfate PG (CS-6PG), after ablation of the sensorimotor cortex of the neonatal and adult rat brain, using 2B6 and 3B3 monoclonal antibodies.

MATERIAL AND METHODS

Neonatal sensorimotor cortical lesion

Three-day-old neonatal Wistar rats of both sexes (1:1) were used in the study. Rats were divided into three groups: sham controls (for each experimental time point) and two groups of rats operated at the third postnatal day (P3) and left for different recovery times (7 and 14 days post-lesion - dpl). All animals were subjected to a 12-h light-dark cycle, with free access to food and water. No more than six pups were kept in the same cage with the mother. Experimental protocols were approved by the Local Animal Care Committee and conformed to the recommendations given in “Guide for the Care and Use of Laboratory Animals” (National Academy Press, Washington, D. C., 1996). Prior to surgery, animals were anesthetized with hypothermia at 14°C. The left sensorimotor cortex was removed by suction ablation through a polypropylene tip. Surgery was performed using the following craniotomy coordinates: 2.5 mm lateral from the midline; and 1 mm anterior and 2.0 mm posterior to the bregma. The position of the sensorimotor cortex was determined using a stereotaxic atlas. The skull was opened on three sides before the surgery, and as soon as the surgery was completed the openings were covered by the incised pieces. The skin was glued using ether solution of collodium. Pups were kept warm until the ether completely evaporated, and then were returned to their mother. Control animals received hypothermic anesthesia and skin cut (sham controls). The sham control rats were littermates of the operated animals. Rats were allowed to recover and sacrificed by decapitation under deep anesthesia after 7 and 14 days.

Adult sensorimotor cortical lesion

The experiments were performed on adult male
Wistar rats weighing 250-300 g. All animals were housed three per cage, with free access to food and water, at constant temperature, humidity, and light (a 12-h light-dark cycle) during the entire experiment. Throughout the experiments, a minimal number of animals was utilized. Prior to surgery, animals were anesthetized with Nembutal 40 mg/kg i.p., shaved, and placed in a stereotaxic frame that the skull was horizontal (i.e., the bregma and lambda were at the same level). The skull was surgically exposed, and coordinates of the left sensorimotor cortex lesion were as follows: 2 mm anterior to the bregma, 4 mm posterior to the bregma, and 4 mm lateral from the midline. Position of the sensorimotor cortex was determined using a stereotaxic atlas. The left sensorimotor cortex was removed by suction ablation to the depth of the white matter. After the lesion, rats were left to recover. A control group of age-matched animals was subjected to the same surgical procedure. Animals were sacrificed under deep anesthesia at days 7 and 14 post-lesion (i.e., 7 and 14 dpi).

**Immunohistochemical evaluation of CSPGs**

Immediately after decapitation, brains were removed from the skull and fixed for 24 h in 4% paraformaldehyde in 0.1 M phosphate buffer (PBS), pH 7.4. Brains were transferred to 10%, 20%, and 30% sucrose in phosphate buffer pH 7.4 (4°C) for cryoprotection, and then frozen in 2-methylbutane pre-cooled to −70°C until cryosectioning on a freezing microtome. Coronal brain sections (16 μm thick) were cut from frontal regions using a cryostat and collected on slides. Immunohistochemistry was performed with two monoclonal antibodies (ICN Biomedicals, Costa Mesa, CA) directed against the 4-sulfate (clone 2B6, an immunoglobulin of the IgG subtype) or the 6-sulfate (clone 3B3, an immunoglobulin of the IgM subtype) stubs of PGs containing chondroitin sulfate type A and B (DS) or C, respectively. The immunoreaction was revealed by the peroxidase-antiperoxidase (PAP) method or the avidin-biotin-peroxidase complex (ABC) (Vectastain ABC kit, Vector Laboratories, Inc., Burlingame, CA, USA) method. In order to test specificity of the reaction, control brain sections were treated in the same way with the omission of primary antibodies. Images were taken with a Leica DMRB photomicroscope.

In all experiments, immunohistochemical results of lesioned animals were compared to sham operated rats of the same age.

**RESULTS**

Immunohistochemistry of sulfated PGs was performed in order to compare the localization and expression profile of chondroitin-4-sulfate PG (CS-4PG) and chondroitin-6-sulfate PG (CS-6PG) in the brain hemisphere after suction ablation of the sensorimotor cortex of the neonatal and adult rat brain. The results were compared with uninjured brain sections prepared from the appropriate control animals (sham control).

**Chondroitin-4-sulfate proteoglycan (CS-4PG)**

**CS-4 expression in the cortex of the control (uninjured) neonatal rat brain**

Immunohistochemical analysis of uninjured-sham control animals revealed low constitutive expression of CS-4 in the cerebral cortex of P10 rats (Fig. 1A). Strong labeling of CS-4 was expressed only on the dorsal surface of the brain, which represents the glia limitans (Fig. 1A, arrow). Light microscope analysis of cortical tissue revealed intense immunolabeling around some neuronal plasma membranes in all cortical layers at P10 (Fig. 1B, arrows) except layer I, which was not immunolabeled (Fig. 1A). However, at P17 extracellularly localized immunoreactivity was expressed throughout all cortical layers, particularly in the glia limitans (Fig. 1D, arrow). Throughout the brain parenchyma, the punctuate manner of CS-4 expression was predominant around single or groups of neurons (Fig. 1E, arrows)

**CS-4 expression after ablation of the sensorimotor cortex of the neonatal rat brain**

After suction ablation of the sensorimotor cortex of 3-day-old neonatal rats, a strong increase of CS-4 labeling was observed in all cortical layers of the lesioned hemisphere, during both investigated periods after the surgery (Fig. 1C, F, G). The appearance of labeling was mainly consistent with extracellular matrix localization. Punctuate labeling around and between neuronal cell bodies was the predominant
Fig. 1. Immunohistochemical detection of CS-4 after unilateral ablation of the sensorimotor cortex of neonatal and adult rats in relation to the uninjured brain. Neonatal brain: (A, B) Cortex of control (uninjured) brain at P10; (C) Lesion site 7 days after lesion; (D, E) Cortex of control (uninjured) brain at P17; (F, G) Injured cortex 14 days after lesion. Adult brain: (H, K) Cortex of control (uninjured) brain; (I, J) Injured cortex 7 days after lesion; (L, M) Injured cortex 14 days after the lesion. Objective magnifications: A, D, F, H, I, L - 10x; G, J, K, M - 20x; B, C, E, - 40x.
Fig. 2. Immunohistochemical detection of CS-6 after unilateral ablation of the sensorimotor cortex of neonatal and adult rats in relation to the uninjured brain. Neonatal brain: (A) Cortex of control (uninjured) brain at P10; (A, inset) Higher magnification of framed area in A; (B) Injured cortex 7 days after lesion; (C) Higher magnification of framed area in B; (D) Cortex of control (uninjured) brain at P17; (E,F) Injured cortex 14 days after lesion. Adult brain: (G, J) Cortex of control (uninjured) brain; (H, I) Injured cortex 7 days after lesion; (K, L) Injured cortex 14 days after lesion. Objective magnifications: A, B, D, E, G, H, K - 10x; I, J, L - 20x; C, F - 40x.
pattern of expression at 7 dpl (Fig. 1C, arrows) and 14 dpl (Fig. 1G, arrows), respectively. Additionally, at 7 dpl some stellate forms of immunoreactivity were observed in the vicinity of the lesion site (Fig. 1C, arrowheads). At 14 dpl, CS-4 expression increases strikingly and strong extracellular staining was seen throughout the whole injured cortex (Fig. 1F). The staining pattern was mostly punctuate (Fig. 1G, arrowheads).

**CS-4 expression in the cortex of the control (uninjured) adult rat brain**

The staining pattern of the 2B6 antibody to CS-4 of adult rats shows a generally low constitutive level of expression in the uninjured brain. Higher expression was seen in subcortical white matter (Fig. 1H, wm). The bulk of CS-4 immunostaining appeared to be extracellularly intercalated between bundles of tightly fasciculate axons running perpendicularly to the pial surface (Fig. 1K, arrows).

**CS-4 expression after ablation of the sensorimotor cortex of the adult rat brain**

Unilateral ablation of the sensorimotor cortex of adult rats provoked strong upregulation of CS-4 expression at 7 dpl (Fig. 1J) and to a lesser extent at 14 dpl (Fig. 1L). Immunostaining was more prominent around the lesion site, where immunoreactive material was arranged in a random unorganized manner (Fig. 1L, I). At 7 dpl, the plasma membranes of neuronal cell bodies were immunopositive (Fig. 1J, arrows). At 14 dpl, extracellularly located material surrounds neuronal cell bodies (Fig. 1M, arrow). In the lesioned area at both 7 dpl (Fig. 1J, J) and 14 dpl (Fig. 1L,M), staining was associated with some stellate, presumably glial cells (black arrowheads) and round, dark-labeled cells, possibly lymphocytes (Fig. 1M, white arrowhead).

Chondroitin-6-sulfate proteoglycan (CS-6PG)

**CS-6 expression in the cortex of the control (uninjured) neonatal rat brain**

The staining pattern of the 3B3 antibody to CS-6 is similar to that obtained for CS-4. In the uninjured brain (sham control), the appearance of labeling was detected at P10 on the surface of neuronal cell plasma membranes. Staining was of the ring type in the form of closed or open circles (Fig. 2A, arrows and inset). During further postnatal development (at P17), immunoreactivity decreased to a low constitutive level. Throughout the brain parenchyma, the extracellular pattern of CS-6 expression was predominant (Fig. 2D).

**CS-6 expression after ablation of the sensorimotor cortex of the neonatal rat brain**

After ablation of the sensorimotor cortex of the neonatal rat brain, CS-6 immunostaining was enhanced in both investigated periods. Seven days after the injury, deposition of CS-6 was observed at the neuronal surface, with protrusion of intensive immunoreactivity into the ECM directed to layer I (Fig. 2B). The cell-associated labeling pattern took the form of a ring, partially or wholly encircling the cell body (Fig. 2C, arrows and inset). Punctuate labeling was observed in the ECM as well (Fig. 2C, arrowheads). However, although the extracellular pattern of CS-6 expression was seen at 7 dpl, the ring type of staining was the predominant pattern. The processes of neuronal cells were generally not labeled for CS-6. In contrast to this, only profound punctuate immunostaining between single or groups of neurons was observed at 14 dpl (Fig. 2F, arrowheads). Strong stellate immunolabeling resembling glial morphology was observed in the vicinity of the lesioned area surrounding the lesion cavity (Fig. 2E, arrows).

**CS-6 expression in the cortex of the control (uninjured) adult rat brain**

The staining pattern of the 3B3 antibody to CS-6 showed a relatively low level of this proteoglycan in the uninjured brain (Fig. 2G, J). The bulk of immunostaining was intercalated between tightly fasciculate axonal fibers oriented to the pial surface (Fig. 2J, arrows).

**CS-6 expression after ablation of the sensorimotor cortex of the adult rat brain**

After ablation of the sensorimotor cortex of the adult rat brain, CS-6 immunostaining was markedly increased at 7 dpl (Fig. 2H) and particularly at 14
dpl (Fig. 2K). The distribution of CS-6 in the injured cortex was more diffuse compared to the neonatal brain. Instead of the punctuate staining detected in the uninjured brain (Fig. 2J), at 7 dpl (Fig. 2H) and especially at 14 dpl (Fig. 2K), abundant deposition of immunoreactivity was seen at the injured site. In close proximity to the lesion site, CS-6 immunoreactivity was arranged in a random unorganized manner (Fig. 2H,K). However, at 7 dpl staining was associated with the neuronal cell bodies as well (in the form of an open circle) (Fig. 2I, arrows). In addition, numerous heavily stained CS-6-positive cells having stellate (glial cells) (Fig. 2I, L, black arrowheads) and round (possibly lymphocytes and macrophages) (Fig. 2K, L, white arrowhead) form surround the lesion site.

DISCUSSION

In this study, the expression pattern of CS-4 and CS-6PG in response to injury was examined using antibodies that recognized the digested stub of sugar dimers sulfated in the 4 or 6 position, which are present in the dermatan sulfate (DS) or chondroitin sulfate types A and C, respectively (Coull et al., 1984). Thus, these monoclonal antibodies reveal the distribution of all proteoglycans containing these types of GAGs. On the basis of immunochemical and biochemical criteria, it would appear that the monoclonal antibodies used in our experiment are monospecific for CSPGs, which have essentially the same size, charge, density, and chemical composition in both the immature and the adult brain and do not react with other brain proteins. These antibodies are therefore useful for comparison of CS-4 and CS-6PG expression during postnatal development and after brain injury in the neonatal and adult rat brain.

Generally, our study showed relative low immunoreactivity of both investigated CSPGs in the early postnatal brain (at P10 and P17), this immunoreactivity being mainly associated with the extracellular matrix (ECM) and membranes of neuronal cell bodies. At P10, strong labeling of CS-4 and CS-6 epitopes was observed only on the dorsal surface of the brain, which represents the glia limitans. This finding is consistent with earlier biochemical and immunochemical data indicating that generally high expression of CSPGs in the embryonic and neonatal brain gradually decreases during postnatal development (Margolis and Margolis, 1993; Fernaud-Espinosa et al., 1996; Stojiljkovic et al., 1998). Although the level of CSPGs is considerably reduced after birth, these molecules are still abundantly detected in regions whose development continues postnatally, regions like the rodent brain, where neuronal migration, differentiation, and neurite outgrowth occur during the first week after birth (Berry et al., 1964; Berry and Rogers, 1965). Our data indicate that in the uninjured brain (sham control) during postnatal development (P10, P17), the expression profile of both investigated CSPGs was similar. We noticed two types of CS-4 and CS-6 immunolabeling — the punctuate type and ring type of staining (the latter wholly or partially encircling the cell bodies) — in all cortical layers except in layer I at P10 for CS-4. Importantly, immunoreactivity of CS-4 and CS-6 on the membrane of neuronal cell bodies is asymmetrically located and oriented in a manner suggesting that it probably represents the sites of primary dendrites, which extend to layer I, a region that contains many fibers, horizontal neurites, and Golgi cells. At P17, CS-4 and CS-6 immunoreactivity was dispersed throughout all cortical layers. Punctuate staining of both CS-4 and CS-6 PG was mostly distributed around single or groups of neuronal cell bodies. This extracellular localization of CSPGs indicates a role for CS-4 and CS-6 in the constitution of perineuronal nets (PNNs), i.e., special structures that comprise a highly condensed matrix surrounding the cell bodies and proximal dendrites of some classes of neurons (Celio et al., 1998; Yamaguchi, 2000). Immunohistochemical studies of the rat brain have shown that the primary sensory area and motor area of the rat cortex contain large numbers of PNNs, which are associated with GABAergic interneurons and certain pyramidal neurons (Hausen et al., 1996; Hartig et al., 1999). It is supposed that the function of CSPGs in PNNs is to prevent unwanted plasticity in normal uninjured adult animals, maintain ion homeostasis, and provide neuroprotection. Also, it is assumed that an increase in CSPG expression within PNNs correlates with the end of a so-called "critical period" of
development and subsequent decrease in plasticity resulting in maintenance of tissue architecture and stabilization of synapses (Galtrey and Fawcett, 2007).

The results presented in this study revealed that the CS-4 and CS-6 expression profile in the neonatal brain is quite distinct and altered compared to the uninjured adult brain. Although most of the immunoreactivity of both investigated CSPGs was associated with an extracellular matrix location, the punctuate pattern of labeling around nerve cell bodies was predominant in the neonatal brain, while the bulk of immunostaining was intercalated between tightly fasciculated axonal fibers running perpendicularly to the pial surface in the adult brain.

Many previous studies have shown upregulation of both CS-4 and CS-6PG after injury to the adult CNS (Mc Keon et al., 1991; Asher et al., 2002; Properzi et al., 2003, 2005; Gilbert et al., 2005). We here demonstrate that cortical ablation provokes upregulation of CS-4 and CS-6 expression not only in the adult, but also in the neonatal rat brain. Enhanced immunoreactivity, seen between 7 and 14 days after the injury, was more prominent around the lesion site, where immunoreactive material was arranged in a random unorganized manner. Moreover, in close proximity to the lesion cavity, stellate forms of immunoreactivity were observed. On the basis of their shape and location, we postulate that these cells are mainly astrocytes. Various lines of evidence suggest that CSPGs are molecules that contribute to glial scarring at the site of CNS injury and represent a family of putative inhibitory ECM molecules that may limit axonal regeneration following injury (Rhodes et al., 2003; Silver and Miller, 2004; Gilbert et al., 2005; Galtrey and Fawcett, 2007). On the other hand, Bicknese et al. (1994) have suggested that CSPGs may play a role in defining discrete axonal pathways during early cortical development rather than acting as a barrier to axonal initiation and outgrowth. Likewise, in previous papers we noticed a close correlation between the absence of glial scar (until 30 dpl) and enhancement of functional recovery after neonatal cortical injury, in contrast with injury to the adult brain, where robust glial scarring was followed by limited recovery (Vasic et al., 1998; Pekovic et al., 2005, 2006). In addition, astrocytes are more growth-supporting in the young than in the adult brain (Smith and Miller, 1991). Thus, it was shown that neurons cultured on immature astrocytes (2-4 days in culture) extended longer neurites at a faster rate than neurons cultured on mature astrocytes (28 days in culture) (Smith et al., 1990), indicating that permissive and non-permissive astrocyte cell types express different levels of CSPGs (Properzi et al., 2003).

In comparison with the normal uninjured brain, the CS-4 and CS-6 expression profile after neonatal cortical injury was totally different and altered following injury. Analysis of the CSPG staining pattern revealed that after neonatal lesion, CS-4 and especially CS-6 were sparsely distributed in the ECM in a punctuate manner (at 14 dpl) and asymmetrically associated with membranes of neuronal cell bodies (7 dpl), in contrast to adults, where cortical lesion induced heavy deposition of staining in the ECM. The question that arises from the obtained data is whether these differences in the pattern of CSPG expression are responsible for better recovery after neonatal vs. adult brain injury? Heavy deposition of CSPG immunoreactivity around the lesion site in adult rats, in contrast to the less CSPG-rich environment in neonatal rats, suggests that enhancement of recovery process after neonatal injury is due to a more permissive environment. Such a permissive environment promotes the recovery process through local axonal growth (collateral sprouting), which occurs not only in the neonatal brain, but also in the adult brain following injury (Li and Raisman, 1995; Silver and Miller, 2004). In denervated regions, growth-associated molecules and cell-adhesion molecules (CAMs), which regulate axonal growth during development, are reacti-vated after the injury and participate in regulation of the sprouting process. In regions of active axonal growth, growth-promoting CAMs are abundant and axonal growth occurs despite the presence of CSPGs (Fukuda et al., 1997). Regenerating axons that grow inwards from the edge of a lesion grow up a gradient of increasingly CSPG-rich ECM. These axons became more branched as they enter
more deeply into the CSGP-rich environment before coming to a complete stop at the center of lesion, indicating that increasing concentrations of CSGPs can promote the formation of a local sprout before acting as a stop signal for elongation. However, this process occurs before a glial scar was established (Davies et al., 1999).

Importantly, it must be taken into consideration that the recovery process in the neonatal brain occurs not only through neurite outgrowth and collateral sprouting, but also through remodelling of brain connections originating from other brain regions, processes where CSGPs have a very important role.

In the light of all these circumstances, our results suggest that not only the amount, but also the pattern of expression of sulfated proteoglycans is an important factor for enhanced recovery after injury to the neonatal vs. the adult brain. Clearly, many questions concerning the mechanisms underlying regulation of expression of ECM molecules in CNS pathology remain to be elucidated.

Acknowledgment — This study was supported by the Ministry of Science of the Republic of Serbia (Grant No. 143005B)

REFERENCES


Rhodes, K. E., Moon, L. D., and J. W. Fawcett (2003). Inhibiting cell proliferation during formation of the glial scar: effects on axon regeneration in the CNS. Neuroscience 120, 41-56.


BRAIN INJURY AND SULFATED PROTEOGLYCANS EXPRESSION

SAJNA DASIC1, SAJNA PECOVIC2, MAJA T. STOJIČKOVIC3, IRENA LAVRČA2, DANIJELA STOJKOV2, IVANA BJELOBABA2 and MIRJANA STOJIČKOVIC2

1Institut za fiziologiju i biohemiju, Biologski fakultet, Univerzitet u Beogradu, 11000 Beograd, Srbija
2Odjećenje za neurobiologiju, Institut za biologiju istraživanja "Sv. Niša, Zavod za istraživanja" 11000 Beograd, Srbija
3ICN Galenika, Biomedicinski razvojni centar, 11000 Beograd, Srbija

Централи нервни систем има ограничен капацитет за опоравак након повреде. Међутим, неонатални мозак показује већу способност опоравка у односу на одрасле. Ове разлике зависе од локалних срединских фактора и степена зрелости аксона током израстања. У групама молекула који могу да стимулишу или инхибирају раст аксона спада и хетероген класа молекула означена као хондроитин сулфатни протеогликан (CSPG). У овом раду испитиван је профил експресије хондроитин-4 и хондроитин-6 сулфатних протеогликана након лезије леве сензомоторне коре неонаталног и адалтног мозга паца."