REVIEW

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Chondroitin sulfate metabolism in the brain

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Over the last twenty years chondroitin sulfate (CS) has become a focus of interest of neuroscience due to its indubitable role in shaping axonal growth, synaptic plasticity and glial scar forming. Various patterns of sulfation give rise to various CS molecules with different properties that are capable of interactions with a plethora of molecules, including growth factors, receptors and guidance molecules. The involvement of CS chains has been implicated in visual critical period regulation, memory formation, spinal cord regeneration. As part of proteoglycan molecules, they are widely expressed in the central nervous system, however, little is known about the enzymatic machinery responsible for CS synthesis and degradation. In this review we attempt to extract and collect the available information concerning the expression and function of enzymes of CS metabolism in the brain.

Key words: chondroitin sulfate metabolism, extracellular matrix, brain, proteoglycans

The extracellular matrix (ECM) of tissues is a non-cellular structure, fundamentally composed of water, proteins and polysaccharides, occupying the space between cells, where the cells secrete these molecules, thus determining the composition and properties of ECM.

ECM is a non-cellular structure, fundamentally composed of water, proteins and polysaccharides. It occupies the intercellular space to which cells secrete various molecules, thus determining the composition and properties of ECM.

It is an important constituent of tissue microenvironment, not only providing mechanical scaffold but also transporting ions and molecules, such as growth factors, chemokines and signaling molecules, affecting cell survival, migration and proliferation and regulating tissue regeneration. It is thought to be crucial in development and aging. The number of pathological syndromes associated with abnormalities in ECM composition implies - its importance in proper functioning of tissues and organs (Järveläinen et al., 2009). Over the last decade, the brain ECM has drawn much attention. The role of ECM molecules has been demonstrated in brain development, critical period plasticity, adult neuroplasticity and regenerative plasticity (Galtrey and Fawcett, 2007). Still, the mechanisms regulating synthesis and modification of brain ECM, as well as the way it regulates brain functioning, are far from understood.

The brain ECM composition and function

Unlike in other tissues, the neural ECM is unique as to the content and architecture. Its striking feature is that it is almost devoid of fibrous proteins, instead it contains large amounts of polysaccharides, both unbound and associated with proteins in the form of proteoglycans (Silbert and Sugumaran, 2002). The matrix of the brain occupies 20% of the brain volume (Sykova and Nicholson, 2008). Apart from water, which is the main component of most tissues, brain ECM contains large amounts of hyaluronic acid (HA), a long linear glycosaminoglycan composed of a repeating disaccharide unit of N-acetylglucosamine (GlcNAc) and glucuronic acid (GlcA) (Csoka and Stern, 2013). The neural ECM also includes chondroitin sulfate proteoglycans (CSPGs) of the lectican family, which are characterized by the capability of interacting with HA. The CSPGs are

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composed of a protein core that is decorated with chondroitin sulfate (CS) chains attached through a covalent bond. CS is a glycosaminoglycan comprised of N-acetylgalactosamine (GalNAc) residues, alternating in ß(1-3) and ß(1-4) glycosidic linkages with glucuronic acid (GlcA). The GalNAc residues are diversely sulfated at the 4- and/or 6-hydroxyl positions and, rarely, the GlcA residue can be sulfated at the 2- or 3-hydroxyl positions (Couchman and Pataki, 2012). Unlike HA, CS chains do not occur in a free unbound form, but are covalently attached through a tetrasaccharide linker, to a serine residue in the protein core of CSPG. The CS chains vary in size up to a hundred or more disaccharide repeating units. Brain CSPGs of the lectican family are represented by aggrecan, versican, neurocan and brevican (Yamaguchi, 2000). The CSPG outside the lectican family is phosphacan - a splice variant of receptor-type protein tyrosine phosphatase (RPTP) that lacks the two intracellular tyrosine phosphatase domains that are found in RPTP (Maurel et al., 1994; Galtrey and Fawcett, 2007). Together with tenascins and link proteins of the HPLN (hyaluronan/HA and proteoglycan binding) family, HA and CSPGs form complex 3-dimensional net-like structures around some neurons, mostly of the gamma-aminobutyric acid (GABA)ergic type (Celio and Blümcke, 1994). The organization of CSPGs in these perineuronal nets (PNNs) during development coincides with critical period closure (Pizzorusso et al., 2002) and CSPGs, as a part of PNNs, have been implicated in limiting plasticity in adult animals (Gogolla et al., 2009; Balmer et al., 2009; Carulli et al., 2010; Slaker et al., 2015). A role for CSPGs in limiting cortical plasticity was confirmed in the studies using chondroitinase ABC (chABC), the enzyme capable of cleaving CS chains. Injection of chABC into the visual cortex reverses the effect of monocular deprivation (Pizzorusso et al., 2006). Injected in superior colliculus, chABC enhances sprouting of retinal afferents after denervation of the superior colliculus resulting from a partial retinal lesion (Tropea et al., 2003). These data support the notion that removal of CS chains from CSPGs molecules allows alterations in the neural circuitry and results in increased plasticity (Fox and Caterson, 2002). Moreover, it appears that the numbers of PNNs, while generally stable in adult brain, can be dramatically decreased upon ischemia, seizures and traumatic brain injury (Hobohm et al., 2005; Harris et al., 2010; Karetko-Sysa et al., 2011; McRae et al., 2012; Yi et al., 2012; Härtig et al., 2017). In view of the above mentioned data, this decrease can be considered as an attempt of the impaired brain to reinstate plasticity in order to allow axonal sprouting and compensation of damaged function (Carmichael et al., 2017). On the other hand, injuries to the central nervous system (CNS) result in upregulation of CSPGs in glial scar, which potently hinders neural plasticity and regeneration (McKeon et al., 1999; Beggah et al., 2005; Harris et al., 2009). For a comprehensive review of CS and CSPGs role in CNS plasticity see Bartus et al. (2012). CSPGs and PNNs have been also suggested to have neuroprotective properties against oxidative stress (Morawski et al., 2004; Egea et al., 2010; Suttkus et al., 2012), the detrimental effect of tau protein (Suttkus et al., 2016), or the effect of β-amyloid (Miyata et al., 2007; Morawski et al., 2010; 2012).

Despite growing evidence of the significance of CSPGs in the CNS, still little is known about CS metabolism in the neural tissue. CSPGs are widely distributed throughout the normal CNS, both during development and in the adulthood and are expressed mostly by neurons and astrocytes (Siebert et al., 2014). However, the molecular machinery responsible for their synthesis and degradation has not been well characterized.

CS biosynthesis

The synthesis of CS is a multistep, enzymatically regulated process, starting in the endoplasmic reticulum (ER) with the synthesis of the xylose (Xyl)-galactose (Gal)-galactose Gal)-glucuronic acid (GlcA) linker from their UDP-precursors (Fig. 1). The four glycosyltransferases involved in the synthesis of the linkage region tetrasaccharide have been molecularly cloned (Mikami and Kitagawa, 2013). The process is initiated by the transfer of Xyl to the hydroxyl group of serine (Sugahara and Kitagawa, 2000). This step is catalyzed by xylosyltransferases, namely xylosyltransferase I and II (encoded by *XYLT1* and *XYLT2* genes, respectively) (Götting et al., 2000). The next step takes place in Golgi apparatus, where β1,4-galactosyltransferase-I (GalT-I, encoded by *B4GALT7* gene) adds the first Gal and β1,3-galactosyltransferase-II (GalT-II, encoded by *B3GALT6* gene) adds the second Gal molecule (Mikami and Kitagawa, 2013). The linker synthesis is completed with addition of GlcA in a reaction catalyzed by a glucuronyltransferase GlcAT-I, encoded by *B3GAT3* gene (Kitagawa et al., 2001a). Additionally, the moieties comprising the linker region can be modified. Transient phosphorylation of Xyl by xylose 2-O-kinase (coded for by *FAM20B* gene) has been described and this modification positively regulates the synthesis of the linker (Oegema et al., 1984; Lohmander et al., 1986; Gulberti et al., 2005; Tone et al., 2008; Koike et al., 2009). Further studies revealed that rapid dephosphorylation of Xyl by 2-phosphoxylose phosphatase (encoded by *PXYLP1* gene) occurs at the stage of GlcA transfer to the linker, just before polymerization begins, and is indispensable for CS polymerization (Koike et al., 2014). It has

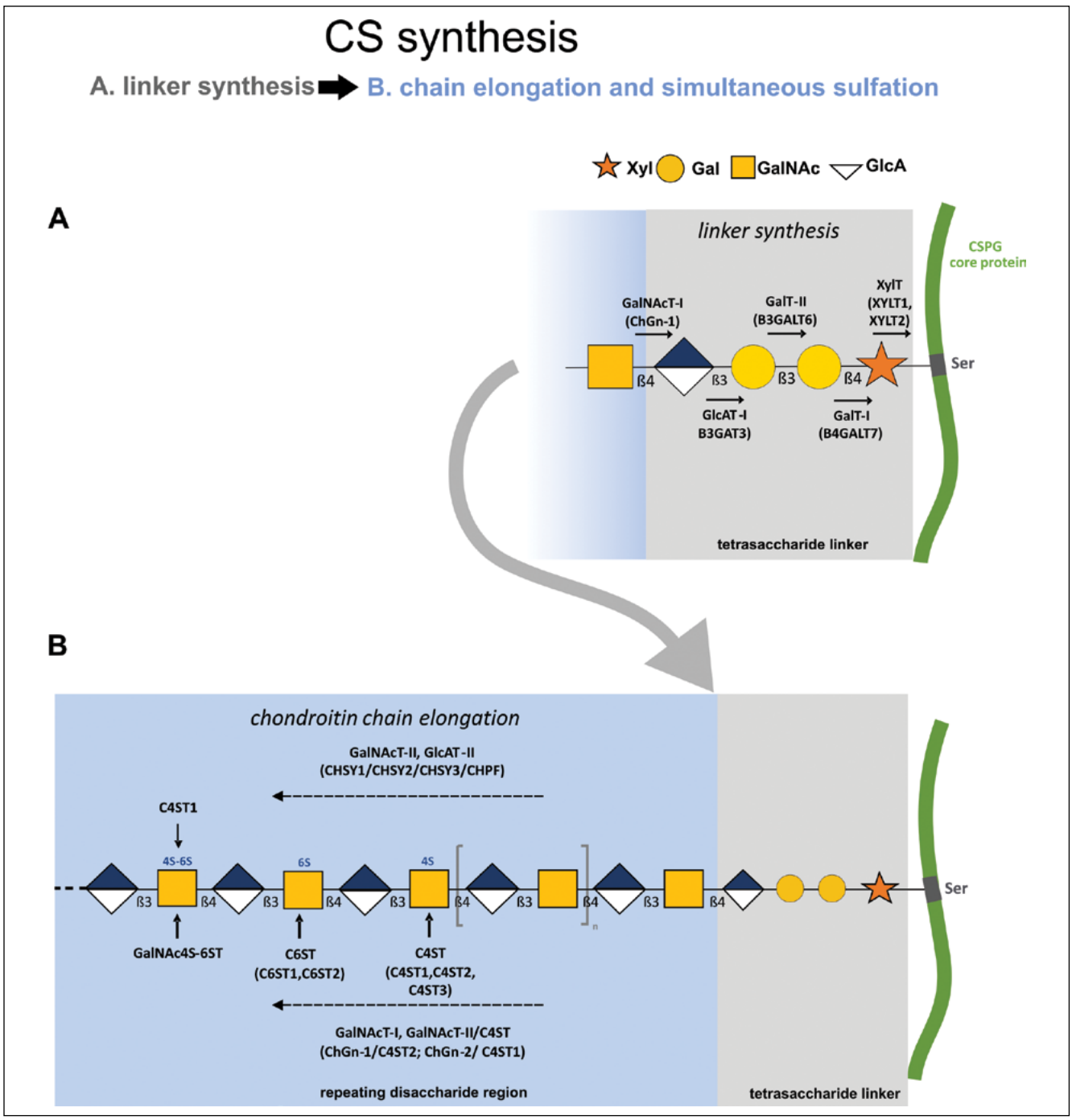

Fig. 1. Chondroitin sulfate biosynthesis pathway. 1: the formation of tetrasaccharide linker; 2: chondroitin chain polymerization and sulfation. The consecutive activities of xylosyltansferase(Xylt), β1,4-galactosyltransferase (GalT-I), β1,3-galactosyltransferase (GalT-II) and β1,3-glucuronyltransferase (GlcAT-I) lead to tetrasaccharide linker formation. Polimerization of the chondroitin chain is initiated by chondroitin GalNAc transferase I (GalNAcT-I) activity and chondroitin chain elongation is catalyzed by chondroitin synthase complex, possesing both N-acetylgalactosamine transferase II (GalNAcT-II) and glucuronic acid transferase II (GlcAT-II) activity, and comprised of chondroitin synthase (ChSy) and chondroitin polymerizing factor (ChPF). Nascent chondroitin chain is modified by sulfation catalyzed by sulfotransferases. An additional pathway of chondroitin polymerization may be executed through cooperation between ChGn-1/2 and C4ST as N-acetylgalactosamine transferase I/ II activity is facilitated by C4 sulfation of CS chain. (B3GALT6) β1,3-galactosyltransferase 6; (B3GAT3) β1,3- glucuronyltransferase 3; (B4GALT7) β1,4-galactosyltransferase 7; (C4ST) chondroitin 4-O-sulfotransferase; (C4ST1-3) chondroitin 4-O-sulfotransferase 1-3; (C6ST1/2) chondroitin 6-O-sulfotransferase 1/2; (ChGn-1) chondroitin N-acetylgalactosamine transferase; (CHPF) chondroitin polymerizing factor; (CHSY1-3) chondroitin synthase 1-3; (CS) chondroitin sulfate; (CSPG) chondroitin sulfate proteoglycan; (Gal) galactose; (GalNAc) N-acetylgalactosamine; (GalNAc4s-6ST) N-acetylgalactosamine 4-sulfate 6-O-sulfotransferase; (GalNAcT-I/II) N-acetylgalactosamine transferase I/II; (GalT-I/II) galactosyltransferase I/II; (GlcA) glucuronic acid; (GlcAT-I/II) glucuronic acid transferase I; (GlcAT-S) glucuronic acid transferase S; (GlcAT-P) glucuronic acid transferase P; (Ser) serine; (Xyl) xylose; (Xylt) xylosyltansferase; (XYLT1/2) xylosyltansferase 1/2).

been also reported that the sulfation at the C-4 and/or C-6 positions of Gal residues markedly influences GalNAcT-I activity of ChGn-1 and its catalytic efficiency (Kitagawa et al., 2008; Gulberti et al., 2012).

The next phase of CS synthesis is chain elongation, starting with a transfer of a single GalNAc to the tetrasaccharide linkage. This step is catalyzed by chondroitin GalNAc transferase I (GalNAcT-I). The first GalNAc transfer triggers the synthesis of the chondroitin backbone (Sugahara and Kitagawa, 2000; Silbert and Sugumaran, 2002). Since the linker region is common to both CS and heparan sulfate (HS), the activity of GalNAcT-I seems to be crucial for differentiating between CS and HS synthesis. Further polymerization of chondroitin proceeds with alternate additions of GlcA and GalNAc residues through the actions of GlcA transferase II (GlcAT-II) and GalNAc transferase II (GalNAcT-II), respectively (Sugahara and Kitagawa, 2000; Silbert and Sugumaran, 2002). So far, six glycosyltransferases involved in chondroitin polymerization have been cloned in humans, namely ChGn-1 (chondroitin GalNAc transferase-1, coded for by *CSGALNACT1* gene) and ChGn-2 (*CSGALNACT2*), ChSy-1 (chondroitin synthase-1, *CHSY1*), ChSy-2 (*CHSY2)*, ChSy-3 (*CHSY3*) and ChPF (chondroitin polymerizing factor, *CHPF*) (Kitagawa et al., 2001b; 2003; Mikami and Kitagawa, 2013).

ChSy-1, ChSy-2, and ChSy-3, possess dual glycosyltransferase activities, GlcAT-II and GalNAcT-II, however, none of them can polymerize chondroitin, unless co-expressed with CHPF (Kitagawa et al., 2001b; 2003; Izumikawa et al., 2007; 2008; Yada et al., 2003). The activity of the complex and the length of produced chondroitin chain depend on the complex composition. Interestingly, ChGn-1 and ChGn-2 reveal both GalNAcT-I and GalNAcT-II activity (Uyama et al., 2002; Gotoh et al., 2002a; 2002b; Sato et al., 2003; Uyama et al., 2003) probably serving as an additional machinery for controlling CS synthesis (for thorough review see Mikami and Kitagawa, 2013).

In addition to heterogeneity resulting from various chain lengths, CS chains display considerable structural variation due to diversified sulfation patterns. CS chains can be modified by sulfotransferases catalyzing the reaction of transfer of the sulfate group from 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to specific sulfation sites on GalNAc or GlcA. Seven sulfotransferases involved in CS sulfation have been cloned so far. Six of them are GalNAc-specific. These are chondroitin 4-O-sulfotransferase-1, 2 and 3 (C4ST-1 - 3), transferring the sulfate group to the C-4 location, chondroitin 6-O-sulfotransferase-1, 2 (C6ST-1 and 2) catalyzing 6-O-sulfation (Fukuta et.al. 1998; Hiraoka et al., 2000; Kitagawa et al., 2000; Yamauchi et al., 2000; Kang et al., 2002), and GalNAc 4-sulfate 6-O-sulfotransferase (GalNAc4S-6ST), which transfers sulfate to the C-6 position of the preexisting 4-O-sulfated GalNAc residue. The seventh sulfotransferase is uronyl 2-O-sulfotransferase (UST), which occasionally sulfates GlcA in the C-2 position of the preexisting 6-O-sulfated GalNAc. Based on the sulfation pattern, several CS disaccharide units have been distinguished: O-unit (GlcA-GalNAc), A unit (GlcA-4SGalNAc), C unit (GlcA-6SGalNAc), E unit (GlcA-4S,6SGalNAc) and D unit (GlcA2S-6SGalNAc) (Fig. 2). The role of GalNAc4S-6ST is also suggested in CS synthesis termination, as the 4,6-O-disulfated GalNAc residue at the non-reducing termini is 60-fold more abundant than in interior disaccharides (Midura et al., 1995).

CS and CSPGs expression in the brain

Since the nervous tissue is recognized as containing large amounts of CSPGs, CS synthesis is presumed to occur widely in most brain cells. Indeed, biochemical analysis of brain tissue homogenates subjected to chondroitinase treatment has revealed substantial amount of CS disaccharides of various types. The anion-exchange high perfomance liquid chromatography (HPLC) analysis shows the predominance of A-unit in adult mouse visual cortex (86.4%) over O-unit (9%), C-unit (2.7%) and E-unit (1.24%) (Hou et al., 2017). Similar data came from the studies of Gilbert et al. (2005), where A-unit accounted for 91% of the total CS amount, followed by O-unit (5%) and C-unit (4%), and those of Carulli et al. (2010) (91.5%, 3.5% and 2.5%, respectively). Various forms of ECM differ in their CS content, e.g. PNNs contain more CS-C and CS-E units and less CS-A units than loose amorphous ECM (Deepa et al., 2006). Different brain regions express CS chains of different structure. A-unit is a major component of CSPGs both in the cerebral cortex and cerebellum, however, the cerebellum is enriched in D-unit, while the cerebral cortex contains a significant amount of E-unit but little D-unit (Maeda, 2010).

The variety of CSPGs has been characterized in rat brain (Rauch et al., 1991; 1992; Jaworski et al., 1994; Gary et al., 2000; Milev et al., 1998; Matthews et al., 2002). Because ECM CSPGs are localized extracellularly, it is not simple to establish the source of origin of specific CSPGs in the nervous tissue. Some answers come from *in situ* hybridization studies or from *in vitro* studies of primary neuronal or astrocytic cultures. *In situ* hybridization on the cerebellar sections from P3 rats revealed the presence of brevican, neurocan, and phosphacan/RPTPbeta transcripts in glial cells, while at P21 brevican could also be found at low levels in some NeuN-positive neurons (Carulli et al., 2006; 2007).

By combining *in situ* hybridization with immunostaining it was shown that neurocan-expressing cells were Gfap-positive and phosphacan/RPTPbeta transcripts were found neural/glial antigen 2 (NG2)–positive cells. In the adult cerebellum, neurocan was found only in neurons (Carulli et al., 2006). Contrastingly, the aggrecan mRNA was detected solely in neurons (Carulli et al., 2006; 2007). Another CSPG of PNNs, versican, and more specifically its most abundant isoform V2, was localized to NG-2-positive cells (Carulli et al., 2007). These results were subsequently confirmed in *in vitro* studies, which showed astrocytic mRNA expression of brevican, neurocan, versican and phosphacan and neuronal expression of aggrecan (Giamanco and Matthews, 2012). As virtually all types of cells in the brain are capable of expressing CSPGs, it is a justifiable assumption that all of them express the metabolic machinery involved in CS synthesis. Little is known, however, about the expression and localization of particular enzymatic proteins in brain regions and cells.

Linker synthesis enzymes in the brain

Data concerning expression and localization of enzymes catalyzing the linker synthesis in the brain are particularly scarce. The activity of XylT enzyme is thought to be ubiquitous across organs and tissues. Both *XYLTI* and *XYLTII* were shown to be expressed in human brain at low levels (Götting et al., 2000; Condac et al., 2009) and in mouse brain (Pönighaus et al., 2007; Yin et al., 2009), as revealed with PCR method. *Xylt1* and *Xylt2* expression was reported in rat cortical astrocytes in primary cultures and Xylt-1 immunoreactivity was

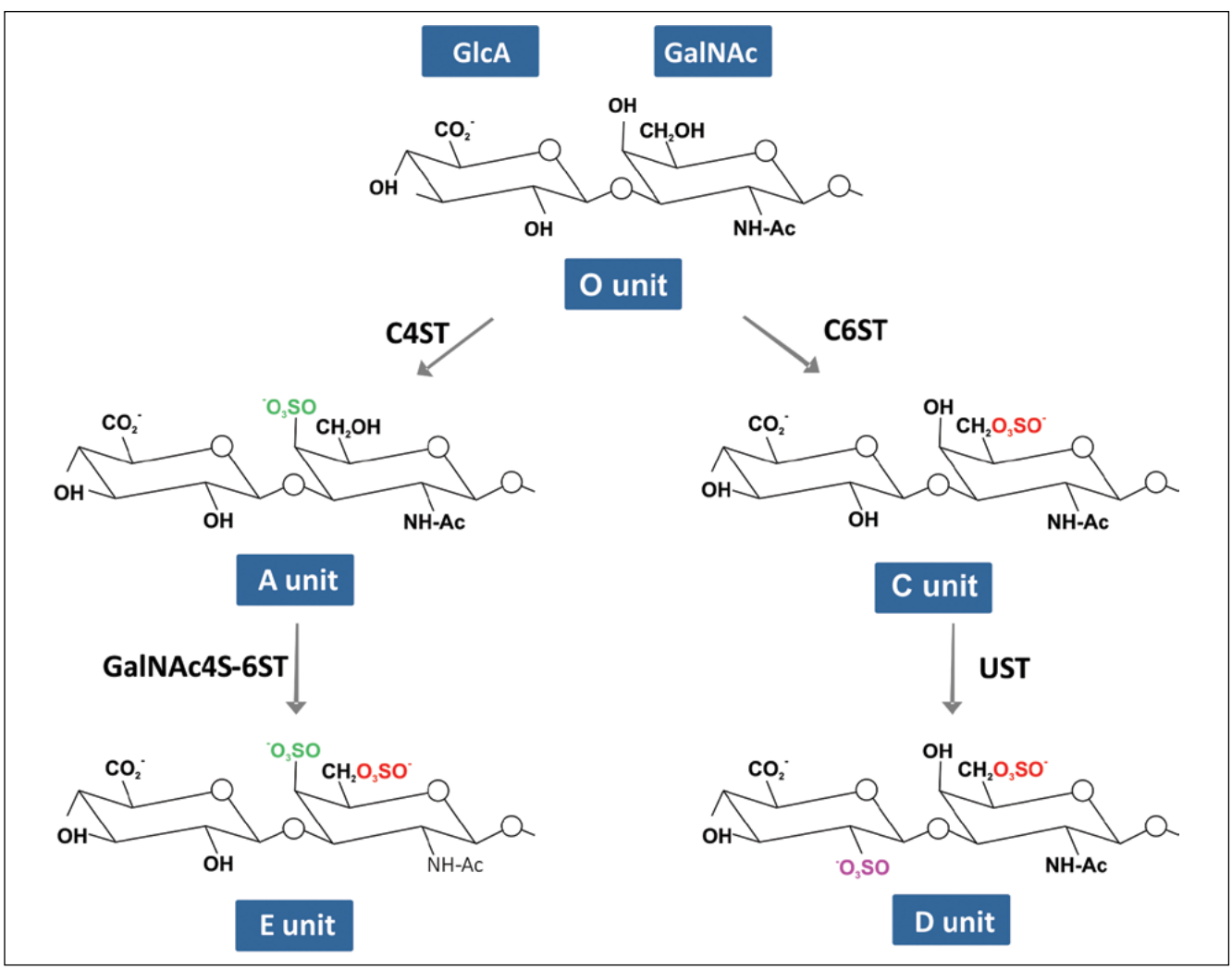

Fig. 2. Chondroitin sulfate (CS) sulfation pathways. C4ST or C6ST activity catalyzes sulfation of chondroitin disaccharide unit. Addition of sulfate group at C4 or C6 position of GalNAc initiates alternative CS chain modification pathways, which may be followed by GalNAc4S-6ST or UST action, respectively. (C4ST) chondroitin 4-O-sulfotransferase; chondroitin 6-O-sulfotransferase; (GalNAc) N-acetylgalactosamine; (GlcA) glucuronic acid; (GalNAc4s-6ST) N-acetylgalactosamine 4-sulfate 6-O-sulfotransferase; (UST) uronyl 2-O-sulfotransferase).

shown in the astrocytes of glial scar after spinal cord injury (Gris et al., 2007). *B4GALT7* expression was reported in human brain using the northern blot method (Almeida et al., 1999). RT-PCR studies revealed the expression of *B4galt7* in mouse cerebral cortex at postnatal day 1 (P1) and in adult animals (8 weeks) (Yin et al., 2009). The brain expression of *B3GALT6* mRNA was determined in human and mouse tissues by northern blot analysis (Zhou et al., 1999; Bai et al., 2001) and RT-PCR (Yin et al., 2009). Another glycosytransferase involved in linker synthesis, GlcAT-I, encoded by *B3GAT3* gene, has been more often studied. The enzymatic activity was first purified from embryonic chick cartilage (Helting and Roden, 1969), and the native protein was partially purified from embryonic chick brain (Brandt et al., 1969). The human gene was cloned (Kitagawa et al., 1998) and further studies using northern blot analysis revealed that it is abundantly expressed in brain (Kitagawa et al., 2001b). Mouse *B3gat3* (GlcAT-I) gene expression in brain was also demonstrated, as early as at embryonic day 7. Notably, in adult mouse brain, *B3gat3* (GlcAT-I) expression was among the highest of all tissues examined (Wei et al., 1999; Kitagawa et al., 2001b; Yin et al., 2009). To our best knowledge, so far, no data have been presented showing cellular or at least regional localization of B4galt7, B3galt6 and B3gat3 protein in animal brain.

CS chain polymerization enzymes in the brain

As mentioned above, Chgn-1 and 2 are the enzymes having both GalNacT-I and GalNacT-II activity and therefore are thought to catalyze initiation and elongation of the chondroitin chain. Despite similarity, the two enzymes apparently have different characteristics and co-operate with different proteins to enhance CS synthesis. It has been shown that ChGn-2 overexpression can positively regulate CS in co-operation with C4ST-1 (Izumikawa et al., 2011) and ChGn-1 more likely cooperates with C4ST-2 to increase the number of CS chains (Izumikawa et al., 2012). In human, both *CSGALNACT1* and *CSGALNACT2* gene (ChGn-1 and ChGn-2 enzyme, respectively) expression was found across brain areas (Uyama et al., 2002; Sato et al., 2003). ChGn-1 transcript presence has been also shown in mouse cerebral cortex (Yin et al., 2009). While virtually nothing is known about ChGn-1 localization in particular cell types in the CNS, it appears that it can regulate the composition of PNNs. It was demonstrated in *ChGn-1* knockout mice that PNN presented diminished Cat-316 epitope, suggesting that it was mainly synthesized in a ChGn-1-dependent manner. The molecular nature and biological role of this epitope hasn't been identified yet, however, recent report showed that Cat-316 antibody preferentially bound to CS chains rich in 4-sulfated disaccharide unit (Yang et al., 2017). Cat-316-positive PNNs selectively bind Otx2, a molecule that is thought to be involved in parvalbumin-positive interneuron maturation and induction of the critical period plasticity (Sugiyama et al., 2008; Miyata et al., 2018). Recently, it has been shown that Otx2 binds to *CSGALNACT1* promoter (Sakai et al., 2017). It appears then, that Otx2 may be directly involved in regulating the synthesis of CS chains (possibly of Cat-316 type) which, in turn, are capable of binding and accumulating Otx2.

The main pathway of CS chain polymerization is mediated by the chondroitin synthase complex, consisting of any two of the four existing synthases, namely ChSy-1, ChSy-2, ChSy-3 and CHPF but not of either one alone (Mikami and Kitagawa, 2013). Different combinations result in different chain lengths, suggesting that the CS chain length is regulated depending on which synthase components are expressed in the cell. Although chondroitin synthases are indispensable for CS and CSPGs synthesis, for unexplainable reasons they have not drawn the attention they deserve. All members of the chondroitin synthase family are expressed in human brain (as visualized by northern blot analysis) (Izumikawa et al., 2008), however, no specific data are available concerning their cellular localization in the nervous tissue. We have come across one study showing *ChSy1* expression in primary cultured microglia (Yin et al., 2009). In mouse brain, *Chpf* was demonstrated to be expressed in two splice variants, A and B, with the B isoform lacking an N-terminal region of 162 amino acids present in A (Ogawa et al., 2010). *In vitro* studies demonstrated that both isoforms can form complexes with Chsy-1 and with each other, however, *in vitro* glycosyltransferase assays reveal no polymerizing activity in ChpfB homodimer complexes and ChpfB/Chs-1 complexes (Ogawa et al., 2010). The presence of Chpf protein was demonstrated in Neu7 astrocyte cell line and in primary astrocytic cultures (Laabs et al., 2007).

Chondroitin sulfation enzymes in the brain

To date, six sulfotransferases involved in CS biosynthesis have been identified (Kusche-Gullberg and Kjellén, 2003). The most typical modification in CS chain is 4-O-sulfation of GalNAc residues, mediated by chondroitin 4-O-sulfotransferases (C4st). Three of them have been cloned and characterized in mammals, namely C4st-1, C4st-2 and C4st-3 (Yamauchi et al., 2000; Hiraoka et al., 2000; Kang et al., 2002). The first two enzymes are broadly expressed. C4st-1 protein/activity was first isolated from the culture medium of rat chondrosarcoma cells (Yamauchi et al., 1999), then

the mouse ortholog was cloned from the brain tissue. Northern analysis indicated that, among various tissues tested, *C4st1* expression was most abundant in mouse brain and kidney (Yamauchi et al., 2000). Human *C4ST1* and *C4ST2* were subsequently cloned and their expression was demonstrated in most brain areas, with highest expression of *C4ST1* in the caudate nucleus, putamen, amygdala and spinal cord, and lower levels in the cortex. *C4ST2* expression was most prominent in the spinal cord, cerebellum, substantia nigra and thalamus and lower levels were found in cortical areas (Hiraoka et al., 2000). The last member of the family, *C4ST3*, presented a more restricted pattern of expression in human tissues. The transcript was found in the liver and kidney and, surprisingly, low level of expression could also be observed in paracentral gyrus of cerebral cortex (Kang et al., 2002) suggesting that *C4ST3* expression might still be present in some restricted brain areas. *C4st1* and *C4st2* expression was demonstrated in astrocyte cell cultures and C4st1 protein was also detected (Properzi et al., 2005; Wang et al., 2008; Yamauchi et al., 2011; Bhattacharyya et al., 2015). Among other sulfotransferases, *C4st1* expression was also observed in neurogenic niches of developing and adult mouse brain, as revealed by *in situ* hybridization (Akita et al., 2008) and in neuronal cultures (Yamauchi et al., 2011). Other *in situ* hybridization studies showed strong *C4st1* expression in the olfactory bulb, striatum, hippocampus and cortex (Sugahara and Mikami, 2007). Although *C4st1* and *C4st2* pattern of expression is to some extent overlapping, it seems that their roles in CS synthesis are not interchangeable. Experimental knockout of *C4st1* results in drastic loss not only in 4-O-sulfation, but also in the amount of CS synthesis (Izumikawa et al., 2011). This suggests that the lack of C4ST-1 activity cannot be compensated by other sulfotransferases and that C4ST-1 plays a crucial role not only in 4-O sulfation but also in regulation of CS biosynthesis (Izumikawa et al., 2011).

Another sulfotransferase involved in CS sulfation, namely chondroitin 6-O-sulfotransferase (C6ST), transfers the sulfate group to the C-6 position of GalNac residue and its activity results in C-unit formation. To date, two isoforms C6ST-1 and C6ST-2 encoded by separate genes (*C6ST1* and *C6ST2*) were cloned in humans and mice (Fukuta et al., 1998; Mazany et al., 1998; Uchimura et al., 1998; Kitagawa et al., 2000). *C6ST1* expression was first demonstrated in brain by northern blot analysis (Fukuta et al., 1998). Likewise, the same method revealed broad expression of *C6ST2* mRNA in many brain areas, with highest levels in the frontal and occipital lobes of cerebral cortex (Kitagawa et al., 2000). In mice, the *C6st1* ortholog was cloned (Uchimura et al., 2002). Knockout studies proved that that 6-O sulfation was compromised in the brain of these animals and C-units decreased by 90% in the brain tissue extracts, similarly to D-units. However, roughly normal brain organization and cortical pyramidal neuron processes were observed (Uchimura et al., 2002). Interestingly, transgenic mice overexpressing C6st-1 present enhanced and prolonged plasticity in the visual cortex pointing to the possibility of plasticity regulation by the C4/C6 ratio (Miyata et al., 2012). Limited data that are available on C6ST-1 expression in CNS suggest widespread localization of enzyme protein in the neural tissue cells. Yin et al. (2009) reported *C6ST1* expression in transforming growth factor (TGF)-β-acivated microglial primary cultures. Low levels of *C6st1* expression were found in neuronal and astrocytic *in vitro* cultures (Yamauchi et al., 2011) and in oligodendrocyte precursor cells and endothelial cells in injured cerebral cortex (Properzi et al., 2005). *C6st1* mRNA expression is developmentally regulated and is much higher at E18 than at P0 and in adult animals (Properzi et al., 2005). Increased C6st-1 immunoreactivity in astrocytes was observed in stab-injured cortex (Okuda et al., 2014). As to C6st-2, its specific localization to cells of the nervous tissue remains to be clearly determined.

GalNAc 4-sulfate 6-O-sulfotransferase (GalNAc4S-6ST), a sulfotransferase transferring the sulfate group to the C-6 position in A-unit is thus responsible for the synthesis of E-unit of CS chains. It was first cloned from human tissue, based on the squid cartilage enzyme amino acid sequence (Ohtake et al., 2001). While no thorough expression analysis was performed for the cloned sequence, it is clear that CNS contains substantial amounts of E-unit-containing CS chains that play a crucial role in regulating axonal growth and neuronal plasticity (Galtrey and Fawcett, 2007). As knockout animals lacking GalNAc4S-6ST expression cannot produce CS containing 4,6-O-disulfated GalNAc residues, it seems that GalNAc4S-6ST is the sole enzyme responsible for this modification (Ohtake-Niimi et al., 2010). This leads to a conclusion that this enzyme is undoubtedly expressed in the CNS. Later *in situ* hybridization studies documented *GalNAc4S6ST* expression during mouse brain development, with ubiquitous expression at P14 and more restricted in adult animals, within olfactory bulb, striatum, cerebellum, dentate gyrus and cerebral cortical layers (Purushothaman et al., 2007). More detailed studies in developing cerebellum showed *GalNAc4S6ST* expression in Purkinje cells, granule neurons, small inhibitory neurons and Golgi neurons (Ishii and Maeda 2008). The *GalNAc4S6ST* expression was also indicated in astrocytic brain tumors (Kobayashi et al., 2013).

Uronyl 2-O-sulfotransferase (UST) catalyzes 2-O-sulfation of the GlcA residue in C unit of CS chains. This modification does occur in CS chains, it is, however, less

frequent (Hou et al., 2016). Human *UST* is expressed in brain (Kobayashi et al., 1999). In mouse brain, *Ust* expression was confirmed by RT PCR and *in situ* hybridization in cells localized in the ventricular zones of the ventral and dorsal telencephalon and in cultured neurospheres (Akita et al., 2008), as well as in differentiated cultured neural cells (Yamauchi et al., 2011). It is downregulated during development and its expression drops significantly between E18 and P0 (Properzi et al., 2005). To date, no published data exist concerning possible significance of CS 2-O sulfation in the nervous tissue. In non-neuronal cells, UST knockdown resulted in inhibition of cell migration (Nikolovska et al., 2015).

CS catabolism

The processes that contribute to CS catabolism are not well understood. However, provided that CS chains are indeed digested by enzymes originally assigned to HA degradation, paradoxically some information can be drawn from HA metabolism studies. The CS degradation is thought to predominantly occur in lysosomes (Prabhakar and Sasisekharan, 2006). CS degradation begins with desulfation of the backbone chondroitin chain by lysosomal sulfatases, arylsulfatase B (coded for by *ARSB* gene) and N-acetylgalactosamine-6-sulfatase (coded for by *GALNS* gene), which remove the sulfate group at the C-4 position and the C-6 position, respectively (Ingmar and Wasteson, 1979; Glössl et al., 1979; Bhattacharyya et al., 2009a). On the basis of similarity of chondroitin backbone with HA, it is presumed, that bare chondroitin chains are degraded in lysosomes by some endoglycosidases and the digestion is continued by exoglycosidases acting at the non-reducing end of the polysaccharide. However, no specific enzymes have been attributed to these processes. It has been reported, however, that some of hyaluronidases, namely HYAL-1, HYAL-4, and SPAM1 (testicular hyaluronidase, PH-20) have CS-degrading activities (Csoka et al., 2001; Jedrzejas and Stern, 2005). It has been shown in *in vitro* studies that HYAL-1 depolymerize CS-A and HA to a similar extent and SPAM1 degrade CS-A, chondroitin, and HA to a similar extent (Csoka et al., 2001; Jedrzejas and Stern, 2005; Honda et al., 2012).

HYAL-4 reveals hydrolytic activity towards CS, however, its expression is restricted to few organs so it cannot be considered responsible for systemic CS catabolism (Kaneiwa et al., 2010; 2012). Further degradation is possibly mediated by ß-hexosaminidase and ß-glucuronidase, the two exoglycosidases that are also responsible for the degradation of HA. Indeed, double knockout mice lacking ß-hexosaminidase activity accumulate excessive CS (Gushulak et al., 2012).

CS catabolism enzymes in the brain

As mentioned above, the CS degradation pathway is poorly recognized and this is true also for the brain. The first step of CS degradation, exclusive for CS and not shared with HA, is desulfation, followed by degradation of desulfated chondroitin backbone with hyaluronidases and exoglycosidases. Desulfation is catalyzed by sulfatases, namely N-acetylgalactosamine 4-sulfatase (arylsulfatase B, ARSB) and N-acetylgalactosamine 6-sulfatase (GALNS). Interestingly, recent studies demonstrated that ARSB is not just a lysosomal enzyme but can also be present at the cell membrane (Bhattacharyya et al., 2009a; 2009b; Mitsunaga-Nakatsubo et al., 2009; Prabhu et al., 2011). ARSB activity and expression was demonstrated in brain and its activity decreased in the ipsilateral hemisphere after unilateral injury (Bhattacharyya et al., 2015). *ARSB* mRNA was also detected in astrocytic cultures (Zhang et al., 2014; Bhattacharyya et al., 2015). GALNS presence in brain is poorly documented. Enzymatic activity was detected in brain extracts (Motas et al., 2016) and in rat primary astrocyte cultures (Zhang et al., 2014).

Once desulfated, the chondroitin backbone is subjected to further degradation by hyaluronidases. Hyal-1 has been proved to have catabolic activity towards CS chains (Honda et al., 2012). While it is thought to be the main hyaluronidase in all tissues (Csoka et al., 2001), when first cloned in humans, its expression could not be confirmed in brain (Frost et al., 1997). Low levels of *Hyal1* expression were found in mouse brain (Shuttleworth et al., 2002). Later studies, however, demonstrated *Hyal1/HYAL1* expression in brain extracts (Al'Qteishat et al., 2006a; 2006b; Xing et al., 2014). HYAL-1 immunoreactivity in stroke patients' brains was found in perilesional and ischemic tissue, mostly in neurons and oligodendrocytes, but not in astrocytes (Al'Qteishat et al., 2006b). Contrastingly, Hyal-1 immunoreactivity was found in astrocytes of rostral migratory stream and subventricular zone in mice and in perilesional cortex of stroke animals (Lindwall et al., 2013).

Hyal-4 is the only CS-specific endo-type hydrolase identified to date, however, its role in CS degradation has not been thoroughly investigated. Human and mouse orthologs differ substantially in their substrate specificities: human HYAL-4 strongly prefers D-unit-rich CS, while mouse enzyme depolymerized A- C- and D-rich CS to a similar extent (Kaneiwa et al., 2012). Hyal-4 expression in brain has not been proved so far. Early studies reported *HYAL4* expression restricted to placenta and skeletal muscle (Csoka et al., 1999). However, Xing et al. (2014) reported low levels of *Hyal4* in rat cerebral cortex of control and injured brain, detected with RT-PCR. Moreover, Hyal-4 protein

expression was documented in spinal cord, after spinal cord transection (Tachi et al., 2015). The enzyme was found in activated fibrous astrocytes of glial scar. These results suggest that, at least under pathological conditions, the cells of the nervous tissue do have the capability of Hyal-4 expression.

Another hyaluronidase possibly involved in CS degradation is PH-20 encoded by *SPAM1* gene (Lin et al., 1993; Jones et al., 1995). Both these early studies showed PH-20 expression strictly limited to testes. However, Sloane and co-workers found that oligodendrocyte precursor cells (OPCs) expressed PH-20, as confirmed by immunocytochemistry (Sloane et al., 2010). Subsequent studies confirmed astrocytic and OPCs PH-20 expression *in vitro* and *in vivo* by immunofluorescence, and transcript presence was revealed by PCR (Preston et al., 2013). *PH20* mRNA was also confirmed in rat brain (Xing et al., 2014). Finally, PH-20 transcript was found detected using nested PCR method and astrocytic PH-20 immunoreactivity was confirmed in ischemically injured white matter in sheep (Hagen et al., 2014). These results were, however, questioned by Marella et al. (2017), who endeavored to replicate the results of Preston et al., and Hagen et al., and claimed lack of PH-20 expression in OPCs cultures in vitro and in the CNS. Clearly, the matter of PH-20 expression and its role in CS (and HA, obviously) degradation in brain requires further extensive investigation.

As hyaluronidases cleave HA/CS to mostly tetrasaccharides, the last step liberating single monosugar moieties is probably mediated by exoglycosidases. These are ß-glucuronidase (encoded by *GUSB* gene) and ß-hexosaminidase (a dimer o two subunits encoded by *HEXA* and *HEXB* genes), which sequentially cleave glycosaminoglycans at a non-reducing end of the chain. The two lysosomal enzymes haven't appealed much to neuroscientists and the number of reports concerning their brain expression or activity is not impressive. Few studies have shown the presence of ß-glucuronidase enzymatic activity in neural cells. It was detected in hippocampal pyramidal neurons and in granule neurons in the cerebellum, and also in granule neurons of the dentate gyrus. ß-glucuronidase localized to perikaryons and dendrites. After brain injury, ß-glucuronidase activity appeared also in glial cells (Vijayan and Cotman 1983). Microglial localization of ß-glucuronidase was reported in the hypoplastic cerebellum of jaundiced Gunn rats (Keino et al., 1990). The activity was also discovered in astrocyte cultures *in vitro*, however, it was restricted to some cells (Kroh and Renkawek, 1973). *Gusb* mRNA expression was found in mouse brain, however at substantially lower levels than in other tissues (Bracey and Paigen, 1987). Gusb protein was detected in brain extracts from rat and monkey (Robins et al., 1968).

Hex genes have been hardly investigated regarding their brain expression. It is disappointing, considering their metabolic implications. Genetic mutation in *HEXA* gene results in ß-hexosaminidase inactivity and causes Tay-Sachs disease, a lethal syndrome including severe neurological dysfunctions and pathological accumulation of metabolites in neurons. In turn, *HEXB* mutations cause Sandhoff disease, resulting in massive accumulation of GM2 ganglioside in CNS (Cordeiro et al., 2000). *Hexa* expression in mice was assessed by northern blot, which revealed the presence of *Hexa* mRNA at substantial levels in brain (Wakamatsu et al., 1994) and *Hexb* expression has been shown to be upregulated after MCAO (Tao et al., 2015). Triple knockouts *Hyal1*$^{-/-}$/*Hexa*$^{-/-}$/*Hexb*$^{-/-}$, resulting in mice deficient in both Hyal1 and ß-hexosaminidase, accumulated substantial amounts of non-sulfated chondroitin in brain (Gushulak et al., 2012). While chondroitin normally is not expressed in brain, it is conceivable that the chondroitin found in the triple knockout tissues may be derived from intermediates of CS metabolism that have already been desulfated by sulfatases (Gushulak et al., 2012).

CS metabolism in brain pathology

It is widely accepted that CSPGs, and their CS chains in particular, are main inhibitory molecules in the CNS limiting neural plasticity. In healthy adult brain, the ECM is rather stable, comprised of large amounts of inhibitory CS-A unit-bearing CSPGs, which make the extracellular space suppressive for the growing processes (Maeda, 2010). However, upon various pathological conditions the structure of ECM changes dramatically in order to create a more plasticity-friendly environment (Hobohm et al., 2005; Karetko-Sysa et al., 2011; McRae et al., 2012; Härtig et al., 2017). On the other hand, during glial scar formation, upregulation of inhibitory CSPGs occurs, preventing axonal growth and regeneration (Kleene and Schachner, 2004) Digestion of CS chains with chABC improves axon regeneration and functional recovery (Bradbury et al., 2002). It appears that the composition of CS chains is regulated upon brain injury. Increased CS-C immunoreactivity was observed around the stab wound injury in cerebral cortex (Properzi et al., 2005; Okuda et al., 2014) and the increase was accompanied with the increase in *C6st1* expression (Properzi et al., 2005). The content of CS-C and CS-E units dramatically increased in injured cortex, and CS-A units were downregulated (Gilbert et al., 2005). In injured spinal cord, CS-A unit content rapidly increases in areas that do not support axonal regeneration (Wang et al., 2008). Similarly, in TBI cortex, an increase in CS-A content, as well as increased immunoreactivity of CS-A was ob-

served in pericontusional tissue (Yi et al., 12). Interestingly, other studies showed that Arsb activity is downregulated and C4st-1 upregulated upon cortical injury (Bhattacharyya et al., 2015) implying the role of the two enzymes in regulating CS-A content and C4/C6 sulfation ratio. The significance of changes in the C4/C6 sulfation ratio in injured brain is still not quite clear. Whereas CS-A unit-rich CS chains are widely accepted to be inhibitory (Wang et al., 2008; Yi et al., 2012; Siebert et al., 2014), there is controversy about CS-C properties as some groups reported it as inhibitory for axonal growth (Snow et al., 1990) while others claim it as permissive (Lin et al., 2011). Similarly, there is no consensus on whether CS-E enhances or impedes neurite outgrowth, it is most often, however, considered inhibitory (Gilbert et al., 2005; Miyata and Kitagawa 2017).

CONCLUSION

There is growing amount of evidence that CS chains of brain ECM play an important role in limitation of neuronal plasticity and nervous tissue regeneration after injuries. Chondroitinase ABC studies proved that modification of CS expression in the tissue is a reasonable approach to facilitating tissue repair. The CS content in brain is changing during development and in pathological conditions and clearly is a subject of enzymatic control. Despite this, remarkably little is known about the regulation of CS synthesis and degradation in the brain. The knowledge concerning enzymes of CS metabolism is scarce and scattered and there are questions that still need answers. What is their localization to specific brain structures and cell types? How is their expression and activity regulated? Considering the enzymatic machinery of CS metabolism a promising target to manipulate, these questions need to be touched upon.

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