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DISTRIBUTION OF EXTRACELLULAR MATRIX MACROMOLECULES IN THE VESTIBULAR NUCLEI AND CEREBELLUM OF THE FROG, *RANA ESCULENTA*

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Abstract—The axons of transected and re-apposed vestibulocochlear nerve of the frog, in contrast to mammalian species, regenerate and establish functional contacts within their original termination areas of the vestibular nuclear complex and the cerebellum. The lack of regenerative capability of the mammalian central nervous system (CNS) is partially attributed to various extracellular matrix (ECM) molecules, such as chondroitin sulfate proteoglycans (CSPG) and tenascin-R (TN-R), which exert inhibition on axon regeneration. In contrast to these molecules, hyaluronan (HA) was reported to be permissive for CNS regeneration. Using histochemical and immunohistochemical methods, we investigated the distribution pattern of these molecules in the medial (MVN), lateral (LVN), superior and descending vestibular nuclei and the cerebellum of the frog and detected regional differences in the organization of the ECM. In the vestibular nuclear complex, pericellular condensation of the ECM, the perineuronal nets (PNNs) were recognizable in the LVN and MVN and were positive only for HA. The neuropil of the vestibular nuclei showed either a diffuse appearance with varying intensity of reactions, or dots and ring-like structures, which may represent the perinodal ECM of the vestibular fibers. In the cerebellum, indistinct PNNs that were only labeled for HA were present in the granular layer. Our findings suggest that the HA-rich, but CSPG and TN-R-free PNNs may be associated with the high degree of plasticity and regenerative potential of the amphibian vestibular system.

Key words: perineuronal net, hyaluronan, chondroitin sulfate proteoglycan, tenascin-R, neural plasticity, brainstem.

INTRODUCTION

The inability of the mammalian central nervous system (CNS) to regenerate remains a major challenge in neurobiology research. In contrast, the regeneration of central pathways, and even some parts of the CNS, is well known in phylogenetically ancestral vertebrates such as various amphibian species and fish (Sperry, 1945; Gaze, 1959; Zakon and Capranica, 1981; Newman et al., 1986; Newman et al., 1987; Becker et al., 1999). Numerous studies have demonstrated a putative role for extracellular matrix (ECM) molecules in CNS regeneration and plasticity following brain and spinal cord injury in various mammalian species, frogs and salamanders (Becker et al., 1999, 2000, 2004; Bradbury et al., 2002; Fox and Caterson, 2002; Pizzorusso et al., 2002; Dityatev and Schachner, 2003; Galtrey and Fawcett, 2007; Halasi et al., 2007; Kwok et al., 2008, 2011; Alilain et al., 2011). In the CNS, the ECM is mainly composed of hyaluronan (HA), proteoglycans and glycoproteins. HA is a glycosaminoglycan which consists of up to 25,000 repeating non-sulfated disaccharide units of glucuronic acid and N-acetylglucosamine. HA, the major component of the embryonic, juvenile and adult CNS, is regarded as a key organizer molecule in the ECM assembly, and is present in sites of cell proliferation and axonal growth (Margolis et al., 1975; Delpech et al., 1989; Yasuhara et al., 1994; Matesz et al., 2005; Carulli et al., 2006; Szigeti et al., 2006; Frischknecht and Seidenbecher, 2008; Meszar et al., 2008; Zimmermann and Dours-Zimmermann, 2008). Each of the proteoglycans possesses a central protein core, which anchors negatively charged, sulfated glycosaminoglycan side chains to form chondroitin sulfate proteoglycans (CSPG) (Kwok et al., 2012). One major molecular group of CSPGs is the lectican family, which consists of four members: aggrecan, versican, neurocan and brevican, each differentiated by core protein length, associated glycosaminoglycan molecules, and degrees of glucuronic acid sulfation of the glycosaminoglycan molecules (Dityatev and Schachner, 2003; Kusche-Gullberg and Kjellen, 2003; Bulow and Hobert, 2006; Galtrey et al., 2008; Zimmermann and Dours-Zimmermann, 2008; Frischknecht and Seidenbecher, 2012; Morawski et al., 2012a). Glycoproteins contain oligosaccharide chains covalently bound to peptides.

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Abbreviations: bHABP, biotinylated hyaluronan-binding protein; bWFA, biotinylated *Wisteria floribunda* agglutinin; CNS, central nervous system; CSPG, chondroitin sulfate proteoglycan; DVN, descending vestibular nucleus; ECM, extracellular matrix; HA, hyaluronan; LVN, lateral vestibular nucleus; MVN, medial vestibular nucleus; NHS, normal horse serum; PNN, perineuronal net; SVN, superior vestibular nucleus; TN-R, tenascin-R; WFA, *Wisteria floribunda* agglutinin.

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The major forms of glycoproteins in the CNS are the laminin, fibronectin, the members of tenascin family i.e. C, R, Y and the link proteins. Via their interactions with other proteins, these ECM molecules form an intricate assembly with HA, bonded to the core protein of CSPGs by link proteins (Dityatev and Schachner, 2003; Galtrey et al., 2008; Zimmermann and Dours-Zimmermann, 2008). ECM molecules are organized into condensed and diffuse forms, and are distributed in an area and neuron-type-dependent manner in the gray and white matter of various species. The condensed form surrounds the perikaryon, dendrites and axon initial segment as the perineuronal net (PNN), or is accumulated at nodes of Ranvier of large and small myelinated axons (Wang and Fawcett; Celio et al., 1998; Dityatev and Schachner, 2003; Carulli et al., 2006; Deepa et al., 2006; Szigeti et al., 2006; Galtrey et al., 2008; Zimmermann and Dours-Zimmermann, 2008; Bekku et al., 2009, 2010; Bekku and Oohashi, 2010; 2012; Giamanco and Matthews 2012). Recently, the axonal coat, another form of condensed ECM associated with preterminal axons and boutons was described in the human basal ganglia, hippocampus and cortex (Brückner et al., 2008; Lendvai et al., 2012, 2013; Morawski et al., 2012a,b; Blosa et al., 2013). The diffuse form of ECM is deposited in the neuropil (Koppe et al., 1997; Carulli et al., 2006). ECM components have important functions in the trafficking of various molecules, and can block or promote cell migration and axonal growth. Anchored to the cell membrane, they are engaged in signal transduction pathways and influence neuronal activity during normal and pathological conditions. In turn, changes in neuronal activity influence the molecular composition of the ECM (Celio et al., 1998; Hartig et al., 1999; Matthews et al., 2002; Dityatev and Schachner, 2003; Sykova and Nicholson, 2008; Dityatev et al., 2010; Morita et al., 2010).

The possible role of ECM molecules in mammalian CNS regeneration has been extensively studied, and the lack of regenerative ability in mammals is partially attributed to the presence of various ECM molecules, such as CSPGs and tenascin-R (TN-R), which inhibit axon regeneration (Becker et al., 2000, 2004; Pesheva and Probstmeier, 2000; Bradbury et al., 2002; Fox and Caterson, 2002; Pizzorusso et al., 2002; Apostolova et al., 2006; Galtrey and Fawcett, 2007; Alilain et al., 2011). In contrast, HA was reported to be a permissive molecule for regeneration of the CNS (Margolis et al., 1975; Preston and Sherman, 2011; Wakao et al., 2011). To gain insight into the possible role of the ECM in neural regeneration in amphibians, the vestibular system of the adult frog is an excellent experimental model. The injured vestibular afferent fibers of the frog, unlike mammals, are capable of morphological regeneration with functional homing of individual axon terminals (Sperry, 1945; Zakon and Capranica, 1981; Newman et al., 1986; Newman and Honrubia, 1992; Dieringer, 1995). In our previous study, after transection and subsequent re-apposition of the eighth cranial nerve of frog, we found temporary reorganization of the PNNs and a decrease of HA reaction density in the medial (MVN) and lateral vestibular nuclei (LVN) (Halasi et al., 2007). After a period of two weeks, both changes gradually receded. The time course of PNN restoration and increase in HA density showed significant differences between the two nuclei. To monitor the possible changes in HA expression during vestibular nerve regeneration in other termination areas of primary afferent vestibular fibers, a detailed analysis of HA distribution in the superior (SVN) and descending vestibular nucleus (DVN) and the cerebellum, is required. Since the PNNs of the mammalian vestibular nuclear complex and the cerebellum contain various CSPGs, TN-R and link protein (Brückner et al., 1993; Bertolotto et al., 1996; Hagihara et al., 1999; Carulli et al., 2006; Deepa et al., 2006; Costa et al., 2007; Morawski et al., 2010; Deak et al., 2012; Rácz et al., 2013), we investigated whether they are expressed in these areas of the frog. In the CNS of non-mammalian species, data on the distribution of ECM molecules are incomplete; activity-dependent formation of CSPG-based PNN was observed in chicken (Domowicz et al., 2003; Morawski et al., 2009; Gáti et al., 2010) and negatively charged surface coats were shown in avian, reptilian, amphibian and piscine brains (Murakami et al., 1994). The present study shows that, unlike mammals, frog PNNs in the LVN, MVN and granular layer of the cerebellum contained high levels of HA but no CSPGs, TN-R or link proteins. These differences between the ECM components of frog and mammalian vestibular nuclear complex may explain the difference in regenerative capability of the vestibular system in different classes of vertebrates.

EXPERIMENTAL PROCEDURES

Animals

The study protocol was reviewed and approved by the Animal Care Committee of the University of Debrecen, Debrecen, Hungary according to national laws and EU regulations [European Communities Council Directive of 24 November 1986 (86/609/EEC)], and was properly carried out under the control of the University's Guidelines for Animal Experimentation (license number: 11/2011/DEMAB). The experiments were performed on adult male and female common water frogs, *Rana esculenta* L. (n = 16; 12 for histochemistry and immunohistochemistry, 4 for immunoblotting). The frogs were drawn from the natural fishpond environment, according to authorized permission (Registration number: OASZF/822/2011/IT:16.18). *Wistar* rats from the Charles River Laboratory (Strain Crl:WI) were used as controls (n = 6; 3 for histochemical or immunohistochemical reactions, 3 for immunoblotting).

Histochemistry and immunohistochemistry

Animal and tissue processing. Frogs (n = 12) were anesthetized with 0.1% ethyl 3-aminobenzoate methanesulfonate salt (MS 222, Sigma–Aldrich, St. Louis, MO, USA), and the animals were perfused