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Glia and volume transmission during physiological and pathological states

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Summary. Extrasynaptic communication between neurons or neurons and glia is mediated by the diffusion of neuroactive substances through the extracellular space (ECS). Structural changes and amino acid release occurring under physiological and pathological conditions result in cellular (particularly glial) swelling, leading to dynamic changes in the ECS volume and geometry that in turn affect ECS diffusion. Significant changes in ECS volume and in diffusion barriers occur during development and aging. They are often the result of cell death, astrogliosis, the rearrangement of astrocytic processes and changes in extracellular matrix molecules. Plastic changes in ECS volume, geometry and anisotropy significantly affect the spatial relation of glial processes towards synapses, glutamate or GABA 'spillover', synaptic cross-talk and neuron-glia communication/interaction. In addition, changes occurring during pathological states can be important for diagnosis, drug delivery and treatment.

Keywords: Anisotropy, apparent diffusion coefficient, extracellular matrix, diffusion-weighted MRI, tortuosity, volume fraction.

Extrasynaptic (''volume'') transmission is mediated by the diffusion of transmitters as well as other substances through the volume of the extracellular space (ECS) (Fuxe and Agnati, 1991; Agnati et al., 1995; Syková, 1997, 2001a; Nicholson and Syková, 1998; Zoli et al., 1999). This mode of communication without synapses provides a mechanism of long-range information processing in functions such as vigilance, sleep, chronic pain, hunger, depression, LTP, LTD, memory formation and other plastic changes in the CNS (for review see Syková, 1997, 2001b, 2002).

Neurons interact both by synapses and by the diffusion of ions and neurotransmitters in the extracellular space. Since glial cells do not have synapses, their communication with neurons is only mediated by the diffusion of ions and neuroactive substances in the ECS. Neurons and glia release ions, transmitters and various other neuroactive substances into the ECS. Substances released non-synaptically diffuse through the ECS and bind to extrasynaptic, usually high-affinity, binding sites located on neurons, axons and glial cells.

The structural changes in the CNS occurring under physiological as well as pathological conditions are accompanied by changes in ECS diffusion parameters. Under physiological conditions this is particularly evident during nervous tissue development (Lehmenkühler et al., 1993; Voříšek and Syková, 1997), during aging (Syková et al., 2002) as well as during lactation (Vargová et al., 2003). Nervous tissue, particularly in the hippocampus and cortex, is subject to various degenerative processes including a decreased number and efficacy of synapses, a decrease in transmitter release, neuronal loss, astrogliosis, changes of astrocytic morphology, demyelination, deposits of beta amyloid and changes in extracellular matrix proteins (for review see Syková et al., 1998, 2002; Syková, 2001b). These and other changes not only affect the efficacy of signal transmission at synapses, but also the function of glia and extrasynaptic or ''volume'' transmission.

Diffusion in the ECS is critically dependent on the structure and physicochemical properties of the ECS – nerve cell microenvironment. These properties vary, however, around each cell and in different brain regions. Certain synapses (''private synapses'') or even whole neurones are clearly tightly ensheathed by glial processes and by the extracellular matrix, so-called perineuronal nets (Celio et al., 1998); others are left more ''naked''. These ''open'' synapses are more easily reached by molecules diffusing in the ECS. On the other hand, many mediators, including glutamate and GABA, bind to high affinity binding sites located on nonsynaptic parts of the membranes of neurons and glia. Mediators that escape from the synaptic cleft at an activated synapse, particularly following repetitive stimulation, diffuse in the ECS and can crossreact with receptors in nearby synapses. This phenomenon, called ''cross-talk'' between synapses by the ''spillover'' of a transmitter (e.g., glutamate, GABA, glycine), has been proposed to account for LTP and LTD in the rat hippocampus (Kullmann et al., 1996; Asztel et al., 1997). The cross-talk between synapses, and the efficacy and directionality of volume transmission, could be critically dependent on the diffusion properties of the ECS.

There is increasing evidence that neuron-glia interactions change, e.g. glia coverage and/or the retraction of glial processes from synapses occur during physiological and pathological functional changes in many brain regions. The glial environment of neurons is likely to be a key factor in the regulation of intersynaptic communication mediated by glutamate. For example, most synaptically released glutamate is taken up by high affinity transporters, such as GLT-1 and GLAST, that are located on surrounding astrocytes (Danbolt, 2001). Moreover, glial cells represent a diffusion barrier in the ECS, hindering the movement of neuroactive substances within the tissue (Nicholson and Syková, 1998; Roitbak and Syková, 1999; Syková, 2001a).

Diffusion parameters of the ECS

The diffusion of substances in a free medium, such as water or diluted agar, is described by Fick's laws. In contrast to a free medium, diffusion in the ECS of the nervous tissue is hindered by the size of the extracellular clefts, the presence of membranes, fine neuronal and glial processes, macromolecules of the extracellular matrix and charged molecules, and also by cellular uptake (Fig. 1A). To take these factors into account, it was necessary to modify Fick's original diffusion equations (see Nicholson and Phillips, 1981; Nicholson and Syková, 1998). First, diffusion in the CNS is constrained by the restricted volume of the tissue available for the diffusing particles, i.e., by the extracellular space volume fraction (α), which is a dimensionless quantity and is defined as the ratio between the volume of the ECS and the total volume of the tissue. It is now evident that the ECS in adult brain amounts to about 20% of the total brain volume, i.e., $\alpha = 0.2$. Second, the free diffusion coefficient (D) in the brain is reduced by the tortuosity factor (λ). ECS tortuosity is defined as $\lambda = (D/ADC)^{0.5}$, where D is the free diffusion coefficient and ADC is the apparent

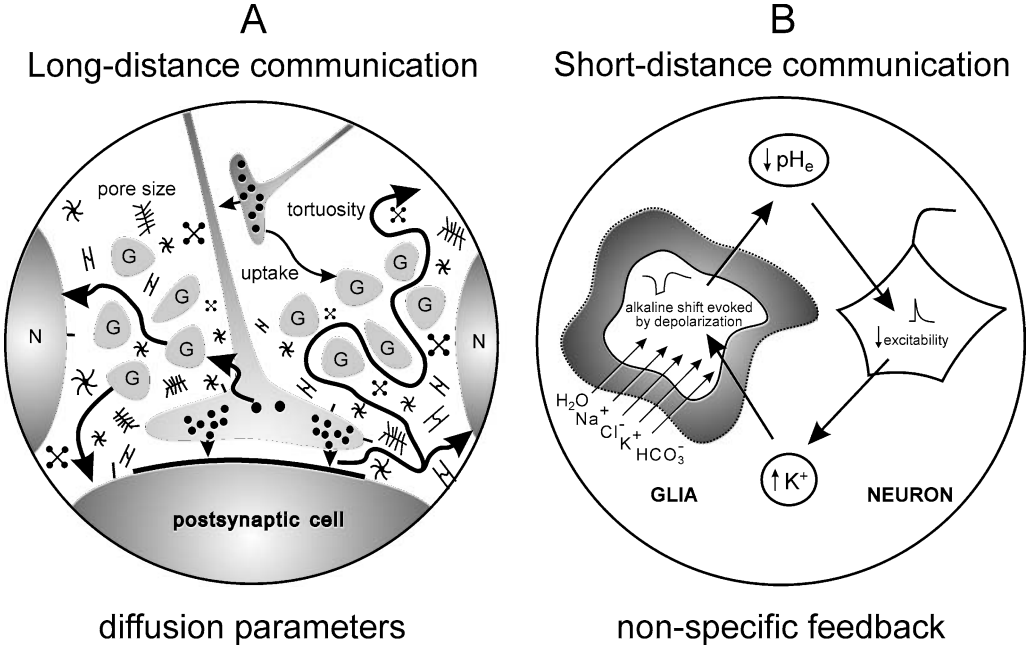


Fig. 1. A Schematic of CNS architecture. The CNS architecture is composed of neurons (N), axons, glial cells (G), cellular processes, molecules of the extracellular matrix and intercellular channels between the cells. The architecture affects the movement (diffusion) of substances in the brain, which is critically dependent on pore size, extracellular space tortuosity and cellular uptake. **B** Schematic of the mechanism of nonspecific feedback suppressing neuronal excitability. Active neurons release K^+, which accumulates in the ECS and depolarizes glial cells. This causes an alkaline shift in glial pH_i and an acid shift in pH_e. Extracellular acidosis further supresses neuronal activity. Transmembrane ionic movements result in glial swelling, ECS volume decrease and therefore in the greater accumulation of ions and neuroactive substances in the ECS

diffusion coefficient in the brain. As a result of tortuosity, D is reduced to an apparent diffusion coefficient $ADC = D/\lambda^2$. Thus, any substance diffusing in the ECS is hindered by membrane obstructions, glycoproteins, macromolecules of the ECM, charged molecules and fine neuronal and glial cell processes. Third, substances released into the ECS are transported across membranes by non-specific concentration-dependent uptake (k'). In many cases however, these substances are transported by energy-dependent uptake systems that obey non-linear kinetics (Nicholson, 2001). When these three factors (α, λ and k') are incorporated into Fick's law, diffusion in the CNS is described fairly satisfactorily (Nicholson and Philips, 1981).

The real-time iontophoretic method is used to determine the ECS diffusion parameters and their dynamic changes in nervous tissue *in vitro* as well as *in vivo* (Syková, 1997; Nicholson and Syková, 1998). Ion-sensitive microelectrodes (ISM) are used to measure the diffusion of ions to which the cell membranes are relatively impermeable, e.g. tetraethylammonium (TEA^+), tetramethylammonium (TMA^+) or choline. These substances are injected into the nervous tissue by pressure or by iontophoresis from an electrode aligned parallel to a double-barreled ISM at a fixed distance. Usually, such an electrode array is made by gluing together an iontophoretic pipette and a TMA^+-sensitive ISM with a tip separation of 130–200 µm. In the case of iontophoretic application, the TMA^+ is released into the ECS by applying a current step of +100 nA with a duration of 40–80 sec. The released TMA^+ is recorded with the TMA^+-ISM as a diffusion curve, which is then transferred to a computer. Values of the ECS volume, *ADC*, tortuosity and non-specific cellular uptake are extracted by a non-linear curve-fitting simplex algorithm applied to the diffusion curves.

By introducing the tortuosity factor into diffusion measurements in nervous tissue, it soon became evident that diffusion is not uniform in all directions and is affected by the presence of diffusion barriers including neuronal and glial processes, myelin sheaths, macromolecules and molecules with fixed negative surface charges. This so-called anisotropic diffusion preferentially channels the movement of substances in the ECS in one direction, (e.g., along axons) and may, therefore, be responsible for a certain degree of specificity in volume transmission. Diffusion anisotropy was found in the CNS in the molecular and granular layer of the cerebellum (Rice et al., 1993), in the hippocampus (Mazel et al., 1998) and in the auditory but not in the somatosensory cortex (Syková et al., 1999b), and a number of studies using the TMA method have revealed that anisotropy is present in the myelinated white matter of the corpus callosum and spinal cord (Vořišek and Syková, 1997; Chvátal et al., 1997; Prokopová et al., 1997). It was shown that diffusion anisotropy in white matter increases during development. At first, diffusion in unmyelinated tissue is isotropic; it becomes more anisotropic as myelination progresses.

The second method that is also currently used to study ECS volume and geometry is diffusion-weighted magnetic resonance imaging (DW-MRI). DW-MRI provides information only about the apparent diffusion coefficient of water (Benveniste et al., 1992; Latour et al., 1994; Norris et al., 1994; Van der Toorn et al., 1996), and a relationship between an increase in the ADC of water and a decrease in ECS volume fraction has recently been found during brain injury

(Voříšek et al., 2002) as well as during pathological aging (Syková, 2001a; Syková et al., 1998, 2000).

ECS diffusion parameters in pathological states

Long-term changes in the physical and chemical parameters of the ECS accompany many pathological states. ''Acute'' or relatively fast changes in the size of the intercellular channels are apparently a consequence of cellular (particularly glial) swelling. An abrupt ECS volume decrease may cause ''molecular crowding'' in the ECS, which can lead to an acute increase in tortuosity. Long-term changes in diffusion would require changes in ECS composition, either permanent changes in the size of the intercellular channels, changes in extracellular matrix molecules, or changes in the number and thickness of cellular (glial) processes. Available data suggest that in some pathophysiological states, α and λ behave as independent variables. A persistent increase in λ (without a decrease in ECS volume) was found during astrogliosis (Roitbak and Syková, 1999; Syková et al., 1999a) and in myelinated tissue (Voříšek and Syková, 1997), suggesting that glial cells can form diffusion barriers, make the nervous tissue less permissive and play an important role in signal transmission, tissue regeneration and pathological states. This observation has important implications for our understanding of the function of glial cells. The extracellular matrix apparently also contributes to diffusion barriers and to diffusional anisotropy, since its loss, e.g. during aging, correlates with a tortuosity decrease and a loss of anisotropy (Syková et al., 1998, 2002).

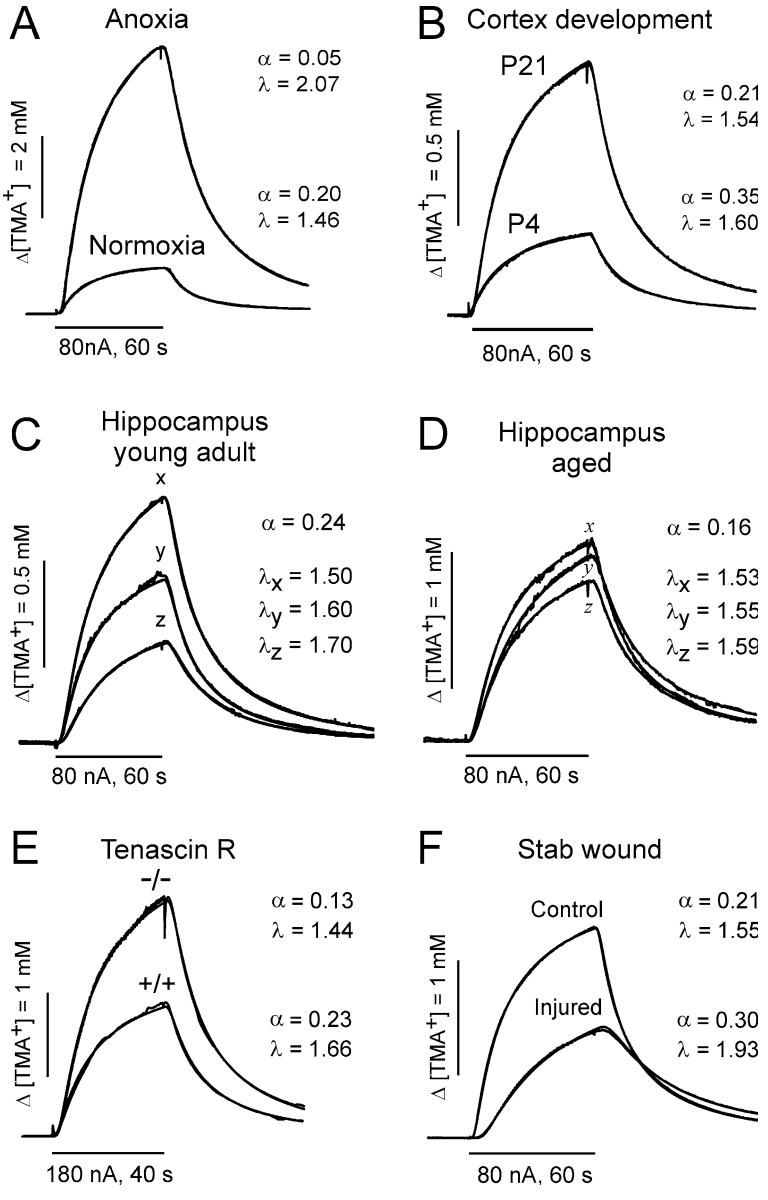
Glia and ECS diffusion parameters

Neuroactive substances, ions and neurotransmitters released during neuronal activity or during pathological states into the ECS interact not only with neuronal membranes at pre- or postsynaptic sites, but also with extrasynaptic receptors, including those on glial cells. Glial cells respond to such stimulation by the activation of ion channels, second messengers and intracellular metabolic pathways. Simultaneously, the cell volume of glial cells increases, including the swelling and rearrangement of their processes, thus causing dynamic variations in the ECS volume. Glial cells, in addition to their role in the maintenance of extracellular ionic homeostasis, may therefore influence extracellular pathways for the diffusion of neuroactive substances.

All transmembrane ionic shifts, e.g. K^+, Na^+, Ca^{2+} and H^+, and membrane transport mechanisms such as glutamate uptake, are followed by water movement, thus causing the shrinkage or swelling of neural cells, particularly glia. Glial cells control ionic and volume homeostasis in the CNS by a variety of mechanisms (for review see Syková, 1992, 1997; Syková and Chvátal, 2000). Besides the Na^+/K^+ pump, ECS K^+ homeostasis is particularly maintained by K^+ spatial buffering and KCl uptake into glia. In depolarized glial cells, an alkaline shift is evoked by activation of Na^+/HCO_3^- cotransport. This alkaline shift in glial pH_i causes an acid shift in pH_e (for review, see Syková, 2001a; Chesler, 2003). Extracellular acidosis, which is a consequence of activity-related extracellular K^+ increase, further suppresses neuronal activity. The following non-specific feedback mechanism suppressing neuronal activity can therefore be proposed in the CNS (Fig. 1B):

1. Neuronal activity results in the accumulation of $[K^+]_e$; 2. K^+ depolarizes glial cells, and this depolarization induces an alkaline shift in glial pH_i; 3. The glial cells therefore extrude acid; and 4. the acid shifts in pH result in a decrease in neuronal excitability by changes due to changes in receptor proteins. Furthermore, since the ionic movements are always accompanied by water, this feedback mechanism would be amplified by activity-related glial swelling, which is compensated for by ECS volume shrinkage. This would result in a greater accumulation of ions and other neuroactive substances in the brain due to the hindrance of their diffusion in the ECS (for review, see Syková, 1997).

In the central nervous system, many pathological processes are accompanied by the loss of nerve cells or their processes, by astrogliosis, manifested as an increase



in glial fibrillary acidic protein (GFAP) staining, by demyelination, and, in addition, by changes in the extracellular matrix. All these processes lead to changes in CNS architecture and may therefore affect the diffusion of neuroactive substances in the ECS. In our department, the mechanisms of the changes in ECS diffusion parameters were studied during many pathological states such as cell swelling evoked by the application of high K^+ or osmotic stress (Vargová et al., 2001; Syková et al., 2003), astrogliosis induced by trauma (stab wound) (Roitbak and Syková, 1999), gliogenesis blocked by early postnatal X-irradiation (Syková et al., 1996), gliosis in tissue grafts (Syková et al., 1999a), demyelination in an animal model of multiple sclerosis – experimental autoimmune encephalomyelitis (Šimonová et al., 1996) and many other degenerative diseases (Fig. 2 – all examples).

Diffusion parameters and the extracellular matrix

The ECS contains a number of glycosaminoglycans (e.g. hyaluronate), glycoproteins and proteoglycans that constitute the extracellular matrix (ECM). Various ECM molecules and adhesion molecules have also been described, e.g. fibronectin, tanescin and laminin, the content of which can dynamically change during development, aging, wound healing and many pathological processes. ECM molecules, produced by both neurons and glia, have been suggested to cordon off distinct functional units in the CNS (groups of neurons, axon tracts, and nuclear groups). These large molecules can slow down the movement (diffusion) of various neuroactive substances through the ECS. Recently, we have shown that Tenascin R-deficient mice have not only a significantly lower tortuosity, but also a

Fig. 2. TMA^+ diffusion curves under different experimental conditions. For each curve, the ECS diffusion parameters α (volume fraction) and λ (tortuosity) were extracted by appropriate non-linear curve fitting. Experimental and theoretical curves are superimposed in each case. For each curve the concentration scale is linear. **A** Typical recordings obtained in adult rat cortex (normoxia) and during anoxia. Note that the larger the curve, the smaller the value of α; a slower rise and decay indicate a higher tortuosity (λ). Values of α are decreased in anoxic tissue, while tortuosity is increased (adapted from Nicholson and Syková, 1998). **B** Typical recordings obtained in rat cortex at postnatal days (P) 4 and 21. Note the dramatic decrease in the ECS volume during maturation (adapted from Nicholson and Syková, 1998). **C** and **D** Diffusion parameters in the hippocampus dentate gyrus of a young adult and an aged rat with memory impairment (rats were tested in a Morris water maze). **C** Anisotropic diffusion in the dentate gyrus of a young adult rat. TMA^+ diffusion curves (concentration-time profiles) were measured along three orthogonal axes (*x* mediolateral, *y* rostrocaudal, *z* dorsoventral). The slower rise in the *z*- than in the *y*-axis and in the *y*- than in the *x*-axis indicates a higher tortuosity and more restricted diffusion. The amplitude of the curves shows that the TMA^+ concentration, at approximately the same distance from the tip of the iontophoresis electrode, is much higher along the *x*-axis than along the *y*-axis and even higher than along the *z*-axis (λ_x, λ_y, λ_z). Note that the actual ECS volume fraction α is about 0.2 and can be calculated only when measurements are done along the *x*-, *y*- and *z*-axes (adapted from Syková et al., 2002). **D** The volume fraction decreases and anisotropy is almost lost in an aged rat with memory impairment. Note that the diffusion curves are higher, showing that α is smaller (adapted from Syková et al., 2002). **E** Values of α and λ are decreased in Tenascin R knockout mice $(-/-)$ (adapted from Syková, 2003). **F** Values of α and λ are increased in the gliotic cortex around a stab wound (injured) (adapted from Roitbak and Syková, 1999)

smaller ECS volume fraction (Syková, 2003). Diffusion-weighted MRI also revealed a decrease in the *ADC* of water in Tenascin-deficient mice (Syková and Vořišek, unpublished data). This suggests that these molecules are important for keeping cellular structures apart, i.e. maintaining the ECS at its optimal size. Furthermore, the optimal size of the ECS volume plays an important role in volume transmission and might play an important role in the synaptic spillover of glutamate and GABA. Indeed, it has been shown that Tenascin-R knockout mice lack perineuronal nets, which leads to lower activity of parvalbumin-containing interneurons and a reduction of perisomatic inhibition of hippocampal pyramidal cells, resulting in increased basal excitatory synaptic transmission and reduced long-term potentiation (LTP) (Bukalo et al., 2001). Importantly, these molecules can hinder the diffusion of molecules so that they are confined to certain places, while diffusion to other brain regions will be facilitated.

ECS diffusion parameters during aging

Morphological changes during aging include cell loss, a loss of dendritic processes, demyelination, astrogliosis, swollen astrocytic processes and changes in the extracellular matrix. It is reasonable to assume that there is a significant decrease in the *ADC* of many neuroactive substances in the aging brain, which accompanies astrogliosis and changes in the extracellular matrix. In aged rats the ECS volume fraction α is lower in the cortex, corpus callosum and the hippocampus, whick correlates with changes in astrocytes and in the extracellular matrix. One of the explanations of why α in the cortex, corpus callosum and hippocampus of senescent rats is significantly lower than in young adults could be astrogliosis. Increased GFAP staining and an increase in the size and fibrous character of astrocytes have been found in the cortex, corpus callosum and hippocampus of senescent rats, which may account for changes in the ECS volume fraction (Syková et al., 1998, 2002). Other changes could account for the decreases in λ values and for the disruption of tissue anisotropy. In the hippocampus in CA1, CA3, as well as in the dentate gyrus, we found changes in the arrangement of fine astrocytic processes. These are normally organized in parallel in the x–y plane, and this organization totally disappears during aging. Moreover, decreased staining for chondroitin sulfate proteoglycans and for fibronectin suggests a loss of extracellular matrix macromolecules.

The degree of learning deficit during aging correlates with changes in ECS volume, tortuosity and non-specific uptake (Syková et al., 2002). The hippocampus is well known for its role in memory formation, especially declarative memory. It is therefore reasonable to assume that diffusion anisotropy, which leads to a certain degree of specificity in extrasynaptic communication, may play an important role in memory formation. There was a significant difference between mildly and severely behaviorally impaired rats (rats were tested in a Morris water maze), which was particularly apparent in the hippocampus. The ECS in the dentate gyrus of severely impaired rats was significantly smaller than in mildly impaired rats. Also, anisotropy in the hippocampus of severely impaired rats, particularly in the dentate gyrus, was much reduced, while a substantial degree of anisotropy was still present in aged rats with a better

learning performance. Anisotropy might be important for extrasynaptic transmission by channeling the flux of substances in a preferential direction. Its loss may severely disrupt extrasynaptic communication in the CNS.

Volume fraction is thus decreasing during the entire postnatal life with the steepest decrease in early postnatal development (Lehmenkühler et al., 1993; Voříšek and Syková, 1997). The larger ECS (30–45%) in the first days of postnatal development in the rat can be attributed to incomplete neuronal migration, gliogenesis, angiogenesis and to the presence of large extracellular matrix proteoglycans, particularly hyaluronic acid, which due to the mutual repulsion of its highly negatively charged branches occupies a great deal of space and holds cells apart from each other. The ensuing decrease in ECS size could be explained by the disappearance of a significant part of the ECM, neuron migration and the development of dendritic trees, rapid myelination and the proliferation of glia. Some of these processes are also observed during aging. The most important are probably neuronal degeneration, a further loss of extracellular matrix and astrogliosis. Indeed, we observed a decrease of fibronectin and chondroitin sulfate proteoglycan staining in the hippocampus of mildly impaired aged rats and almost a complete loss of staining in severely impaired aged rats. Chondroitin sulfate proteoglycans participate in multiple cellular processes, e.g. axonal outgrowth, axonal branching and synaptogenesis, which are important for the formation of memory traces (Hardington and Fosang, 1992; Margolis and Margolis, 1993). The observed changes in ECS diffusion parameters during aging may therefore have an important functional significance. Anisotropy, which, particularly in the hippocampus and corpus callosum, may help to facilitate the diffusion of neurotransmitters and neuromodulators to regions occupied by their high affinity extrasynaptic receptors, might have crucial importance for the specificity of signal transmission. The observed loss of anisotropy in senescent rats could therefore lead to impaired cortical and, particularly, hippocampal function. The decrease in ECS size could be responsible for the greater susceptibility of the aged brain to pathological events, the poorer outcome of clinical therapy and the more limited recovery of affected tissue after insult.

It can be concluded that the diffusion of neuroactive substances is hindered not only by the size of the pores between the cells, but also by the cellular structure, including the fine swelling and movement of glial processes towards active synapses, and changes in extracellular space diffusion barriers – extracellular matrix molecules. The extracellular matrix, besides its apparent importance for tissue anisotropy, is also important for maintaining a relatively large ECS volume. The changes in ECS diffusion parameters in physiological and pathological states can affect the efficacy of synaptic as well as extrasynaptic transmission, induce damage to nerve cells by the increased accumulation of toxic substances, as well as be an important factor to consider when using diffusion-weighted MRI for diagnostic purposes or when considering therapeutic drug application.

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