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Extracellular diffusion in laminar brain structures exemplified by hippocampus

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Abstract

Numerous brain structures are composed of distinct layers and such stratification has a profound effect on extracellular diffusion transport in these structures. We have derived a more general form of diffusion equation incorporating inhomogeneities in both the extracellular volume fraction (α) and diffusion permeability (θ). A numerical solution of this equation for a special case of layered environment was employed to analyze diffusion in the CA1 region of hippocampus where stratum pyramidale occupied by the bodies of principal neurons is flanked by stratum radiatum and stratum oriens. Extracellular diffusion in the CA1 region was measured in vitro by real-time iontophoretic and real-time pressure methods, and numerical analysis found that stratum pyramidale had a significantly smaller extracellular volume fraction ($\alpha = 0.127$) and lower diffusion permeability ($\theta = 0.327$) than the other two layers ($\alpha = 0.218$, $\theta = 0.447$). Stratum pyramidale thus functioned as a diffusion barrier for molecules attempting to cross it. We also demonstrate that unless the detailed properties of all layers are taken into account when diffusion experiments are interpreted, the extracted apparent parameters of the extracellular space lose their physical meaning and capacity to describe any individual layer. Such apparent parameters depend on diffusion distance and direction, giving a false impression of microscopic anisotropy and non-Gaussian behavior. This finding has implications for all diffusion mediated physiological processes as well as for other diffusion methods including integrative optical imaging and diffusion-weighted magnetic resonance imaging.

Keywords

extracellular space; volume fraction; diffusion permeability; tortuosity; ion-selective microelectrodes; real-time iontophoretic method; tetramethylammonium; numerical modeling

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1 Introduction

Brain cells are surrounded by the extracellular space (ECS) which facilitates diffusion of neurotransmitters, neuromodulators, nutrients, metabolites and therapeutic agents. The ECS therefore serves as an important functional counterpart of neurons and glia. While the morphology of neurons and glia is well known, the overall structure of the ECS is much less understood (Sykova and Nicholson, 2008). The ECS channels are too narrow to be resolved by light microscopy. At the same time, the ECS structure is too vast and complex to be reconstructed in three dimensions from electron micrographs, which also requires sample preparation known to affect ECS volume (Van Harreveld et al., 1965; Sykova and Nicholson, 2008).

Most of our knowledge about the ECS in living brain tissue originates from diffusion studies that deduce structural parameters of the ECS from diffusion of extracellular marker molecules (Nicholson, 2001; Hrabetova and Nicholson, 2007; Sykova and Nicholson, 2008). The region of interest under investigation has always been assumed to be homogeneous. This is a valid assumption in the majority of cases because typical spatial resolution in the ECS diffusion experiments is quite high, usually about $100\ \mu\text{m}$. However, there are important circumstances where morphology varies over such short distances, e.g., in the layered structures of hippocampus or retina, across the gray-white matter boundaries, or across the adjacent nuclei.

We will show that, when a more realistic mathematical model of the layered structure is constructed, it is possible to extract parameters of the individual layers and explain experimental data obtained from measurements across the layers. With such a model in place, we were able to predict the result of diffusion measurements in a direction parallel to the layers, and verify this prediction experimentally. We will also show that if the inherent inhomogeneity of these structures is not taken into account, it can lead to inaccurate values of the estimated diffusion parameters and the microscopic anisotropy indices derived from them.

Diffusion experiments quantify two macroscopic ECS parameters. One parameter is the ECS volume fraction α , defined as a proportion of the tissue volume occupied by the

ECS ($\alpha = \frac{V_{\text{ECS}}}{V_{\text{tissue}}}$). Another parameter, diffusion permeability θ , quantifies how much the diffusion process in a complex ECS environment slows down in comparison to an obstacle-free medium. It is defined as $\theta = \frac{D_{\text{ECS}}}{D_{\text{agar}}}$, where D_{ECS} and D_{agar} are the diffusion coefficients measured in brain and in an approximation of free medium, respectively (Hrabe et al., 2004). Alternatively, diffusional hindrance can be described by tortuosity, usually (but not always) defined as $\lambda = \frac{1}{\theta}$ (Nicholson and Phillips, 1981). However, precise physical interpretation of tortuosity is not as straightforward as diffusion permeability, and it does not depend linearly on the timing of physiological signals mediated by diffusion.

From a functional point of view, α determines the concentration of molecules released into the ECS. The same amount of substance released into a smaller ECS volume results in higher concentrations, and vice versa. Diffusion permeability θ determines the timing of mediated signals. Lower values lead to correspondingly slower diffusion rates and thus longer diffusion times, while higher values indicate proportionally faster rates and shorter times. A typical value of α obtained with small probe molecules in isotropic and homogeneous brain regions is 0.2, which means that approximately 20% of brain tissue is taken up by the ECS. A typical value of θ is 0.4, that is, the rate of diffusion through ECS is

only about 40% of the rate observed in a medium free of any obstacles (Sykova and Nicholson, 2008).

The layered structure we examined is found in the rat hippocampal CA1 region where the bodies of principal neurons are tightly packed in stratum pyramidale (SP), flanked by stratum radiatum (SR) and stratum oriens (SO) (Figure 1a). The SP layer is fairly thin, only about 50 μm (Turner et al., 1991; Lopez-Aguado et al., 2001). The diffusion properties were measured with well-established real-time iontophoretic (RTI) method (Nicholson and Phillips, 1981; Sykova and Nicholson, 2008), employing a small extracellular marker molecule tetramethylammonium (TMA^+) (Kaur et al., 2008). The RTI method is uniquely applicable to this task because it is sensitive to both α and θ parameters. The SP layer effectively functioned as a barrier, preventing many molecules diffusing across it from reaching the other side. On the other hand, molecules diffusing over comparable distances within the SP could do so with a smaller drop in concentration.

2 Materials and Methods

2.1 Rat brain slices

Diffusion experiments can be performed both in a brain of an anesthetized animal and in an acute brain slice. In general, there is a good agreement in the ECS parameters obtained in these two preparations (Sykova and Nicholson, 2008) and therefore the results obtained in either of these two preparations have general applicability. As described in detail elsewhere (Hrabetova and Nicholson, 2007), each preparation offers certain advantages and the choice for a particular study depends on the brain region under investigation and on the type of experiment. Here we selected the brain slice preparation primarily because the position of both microelectrodes with respect to the individual hippocampal layers can be controlled with great precision.

All experiments were conducted at the NYU School of Medicine in accordance with NIH guidelines and local Institutional Animal Care and Use Committee regulations. Coronal brain slices 400 μm thick were prepared from adult Sprague-Dawley female rats (175–250 g) as described previously (Hrabetova, 2005; Hrabetova et al., 2009). The animals were anesthetized with sodium pentobarbital (50 mg/kg I.P.) and decapitated. The brain was extracted, cooled with ice-cold artificial cerebrospinal fluid (ACSF) and sliced with a vibrating blade microtome (VT 1000 S; Leica Instrument GmbH, Nußloch, Germany). The slices (Figure 1a) were held at room temperature, submerged in the ACSF in an incubation chamber. The composition of ACSF was as follows (in mM): NaCl 124, KCl 5, NaHCO_3 26, NaH_2PO_4 1.25, D-glucose 10, MgCl_2 1.3, CaCl_2 1.5. The ACSF was gassed with a mixture of 95% O_2 and 5% CO_2 to buffer the pH at 7.4. The ACSF osmolality, determined with a freezing point-depression osmometer (Osmette A #5002; Precision Systems Inc., Natick, MA), was approximately 300 mosmol/kg. A small amount of TMA^+ chloride (0.5 mM) was added to the ACSF to provide a reference concentration.

2.2 Diffusion measurements

For diffusion measurements, either dilute agarose gel (0.3% in 150 mM NaCl; NuSieve GTG, FMC BioProducts, Rockland, ME) or a brain slice were placed in a submersion recording chamber (model RC-27L; Warner Instrument Corp., Hamden, CT). The specimen was super-fused with ACSF at a flow rate of 2.0 mL/min delivered by peristaltic pump. The ACSF temperature was maintained at $34 \pm 1^\circ\text{C}$ by a dual automatic temperature controller (model TC-344B; Warner Instrument Corp.) operating an in-line heater (model SH-27A; Warner Instrument Corp.) in tandem with the heating element of the recording chamber. The recording chamber was placed on the fixed stage of a compound microscope (BX61WI;

Olympus America, Melville, NY) equipped with a water immersion objective (Olympus UMPlanFI, 10 $\times$, numerical aperture 0.3) and infrared differential interference contrast optics for visualization of the specimen and the microelectrodes (Figure 1b). The glass micropipettes for delivery and detection of the diffusion probe TMA⁺ were held in two robotic micromanipulators (MP 285; Sutter Instruments, Novato, CA) which ensured high precision of movement and stability.

Two complementary methods, the real-time iontophoretic (RTI) method and the real-time pressure (RTP) method, were employed in this study to measure diffusion of the TMA⁺ probe molecules. We will describe them only briefly as the theory and algorithms for analysis can be found elsewhere (Nicholson and Phillips, 1981; Nicholson and Rice, 1987; Nicholson, 2001; Hrabetova and Nicholson, 2007).

2.3 Real-time iontophoretic (RTI) method

Tetramethylammonium (TMA⁺, MW 74) was released by iontophoresis from a microelectrode and detected by an ion-selective microelectrode (ISM) positioned about 100 μ m away from the source. Microelectrodes were prepared from double-barreled theta glass (Warner Instrument Corp.) as described in detail by Nicholson (1993). Both barrels of the iontophoretic microelectrode were filled with 150 mM TMA⁺ chloride. Continuous positive bias current of 20 nA was applied from a constant current, high impedance source (Axoprobe-A1 Amplifier; Axon Instruments, Inc., Union City, CA) to maintain a steady transport number (n_t) throughout the experiment (Nicholson and Phillips, 1981). Diffusion curves were obtained by stepping up the iontophoretic current to 60–120 nA for 50 s.

The TMA⁺ detecting barrel of the ISM was filled with 150 mM TMA⁺ chloride and contained a short column of tetraphenylborate based exchanger in the tip (Corning exchanger 477317; currently available as IE 190 from WPI, Sarasota, FL). The reference barrel, which detected DC potential, was filled with 150 mM NaCl. Each ISM was calibrated in a set of standard solutions (0.5, 1, 2, 4, and 8 mM TMA⁺ in 150 mM NaCl). Calibration voltages were fitted to the Nikolsky equation to obtain the slope and the interference of each ISM (Nicholson, 1993).

In the recording setup, the iontophoretic microelectrode and the ISM were independently positioned in the agarose gel or the brain slice (Hrabetova et al., 2002). Slice measurements were made in the center of the slice (i.e., at a depth of 200 μ m) to minimize the effect of tissue-ACSF interface (Hrabetova and Nicholson, 2007). The TMA⁺ signal was obtained as a difference between potentials of the detecting barrel and the reference barrel, using a dual-channel microelectrode preamplifier (model IX2-700; Dagan Corp., Minneapolis, MN). The signal was amplified and low-pass filtered (6 Hz) using a CyberAmp 320 (Axon Instruments, Inc.), then digitized and stored on a personal computer and monitored on an analog chart recorder (Hrabetova and Nicholson, 2007).

Diffusion curves obtained with the RTI method in dilute agarose gel or brain slices were fitted with a solution of the diffusion equation (Nicholson and Phillips, 1981) using a Matlab (MathWorks, Natick, MA) based program (Hrabetova and Nicholson, 2007). Measurements in the agarose gel yielded the transport number n_t of the iontophoretic microelectrode and the diffusion coefficient D_{agar} (Figure 1c). Dilute agarose gel is used to minimize thermal convection currents known to cause inaccuracies when water or physiological solutions are used (Nicholson, 2001). The D_{agar} measured under these experimental conditions is essentially a free diffusion coefficient because 0.3% agarose gel reduces diffusion coefficient of a small ion only negligibly (less than 0.7%; Slade et al., 1966).

To verify stability of the transport number throughout the experiment, additional measurements in the agarose gel were made after recording in the brain slices. Once these parameters were known, measurements in the brain slices provided estimates of the ECS volume fraction α , diffusion permeability θ , and nonspecific clearance (Figure 1c; Nicholson and Phillips, 1981; Nicholson, 1992).

2.4 Real-time pressure (RTP) method

TMA⁺ was released by pressure pulse from a microelectrode and detected by an ISM positioned about 100 μm away from the source. Microelectrodes for pressure injection were fabricated from glass micropipettes pulled from thin wall glass tubing (catalog #6170; A-M System, Inc., Carlsborg, WA) and filled with 150 mM TMA⁺ chloride. For diffusion measurement a brief pressure pulse of nitrogen was applied (duration 5–50 ms, pressure 20 psi). The ISMs were prepared and calibrated as described for the RTI method. Signal amplification, filtering and recording was also identical.

ECS parameter estimates are obtained similarly to the RTI method (Figure 1d; Nicholson and Rice, 1987). However, it is not possible to make an accurate estimate of a parameter analogous to the transport number n_t , and consequently the ECS volume fraction α cannot be obtained by the RTP method.

2.5 Theory and numerical model

The parabolic partial differential equation governing diffusion was originally derived for homogeneous and isotropic environments free of any obstacles. It can be shown that under certain conditions the same equation macroscopically describes diffusion in a complex environment with obstacles and pores (Lehner, 1979; Hrabe et al., 2004). It can also easily accommodate anisotropic and spatially variable diffusion permeability tensor $\theta = \frac{D_{\text{ECS}}}{D_{\text{agar}}}$, and thus a spatially variable diffusion tensor $\mathbf{D}_{\text{ECS}}$. However, the diffusion coefficient is not the only source of inhomogeneity. Another possibility is that the volume fraction $\alpha = \frac{V_{\text{ECS}}}{V_{\text{issue}}}$ varies in space. Here we derive a generalized diffusion equation covering both sources of inhomogeneity, assuming that Fick's first law is still macroscopically valid for the extracellular compartment.

Let $c = c(\vec{r}, t)$ be the ECS concentration of a diffusing substance at position $\vec{r} = (x, y, z)$ and time t . If $\alpha = \alpha(\vec{r})$ is the ECS volume fraction, the total amount of a substance Q in some small tissue volume V is

$$Q = \int_V \alpha(\vec{r}) c(\vec{r}, t) dV.$$

The macroscopic ECS diffusion flux $\vec{j}$ is driven by the ECS concentration gradient,

$$\vec{j} = -D_{\text{agar}} \theta \cdot \nabla c,$$

where $\nabla = (\partial_x, \partial_y, \partial_z)$ is the gradient operator and dot signifies the inner product. This is Fick's law for a geometrically complex environment. However, because flux in the tissue is defined as the amount of substance per unit time crossing a unit surface area, we need to know what percentage of surface area within tissue intersects ECS. The Delesse principle (Howard and Reed, 1998) states that this percentage is equal to the ECS volume fraction α ,

and the tissue diffusion flux is therefore reduced from $\vec{j}$ to $\alpha\vec{j}$. This is valid even in a locally anisotropic environment as long as the surface orientation varies with respect to the axes of anisotropy, which is fulfilled automatically when the surface encloses a small representative volume of no special shape. The law of conservation of mass for a small tissue volume V with surface S can now be applied in the usual way, taking advantage of Gauss's divergence theorem:

$$\frac{\partial Q}{\partial t} + \oint_S \alpha \vec{j} \cdot d\vec{S} = \int_V s dV$$

$$\int_V \frac{\partial \alpha c}{\partial t} dV = D_{\text{agar}} \int_V \nabla \cdot (\alpha \theta \cdot \nabla c) dV + \int_V s dV,$$

where $s = s(\vec{r}, t)$ is the source strength, defined as the amount of substance delivered per unit time into unit volume of tissue. We thus obtain a generalized version of the diffusion equation for an anisotropic environment where both the diffusion permeability tensor and the volume fraction are inhomogeneous:

$$\frac{\partial c(\vec{r}, t)}{\partial t} = \frac{D_{\text{agar}}}{\alpha(\vec{r})} \nabla \cdot \left[\alpha(\vec{r}) \theta(\vec{r}) \cdot \nabla c(\vec{r}, t) \right] + \frac{s(\vec{r}, t)}{\alpha(\vec{r})}. \quad (1)$$

A simplified version of Eq. 1 was mentioned previously by Hrabetova et al. (2009). Alternatively, we can rewrite it in terms of tissue concentration $\tilde{c} = \alpha c$ as

$$\frac{\partial \tilde{c}}{\partial t} = D_{\text{agar}} \nabla \cdot \left(\alpha \theta \cdot \nabla \frac{\tilde{c}}{\alpha} \right) + s, \quad (2)$$

which is a form relevant for integrative optical imaging or diffusion sensitized magnetic resonance spectroscopy because these methods detect $\tilde{c}$ rather than c . A corresponding version of Fick's first law for tissue flux $\vec{j}$ is

$$\vec{j} = -D_{\text{agar}} \alpha \theta \cdot \nabla \frac{\tilde{c}}{\alpha}.$$

Under most circumstances, Eq. 1 has to be solved numerically. However, it is instructive to consider a special case of a steady state diffusion where the time derivative vanishes. The diffusion then behaves exactly as if the diffusion tensor was equal to $D_{\text{agar}} \alpha \theta$. In other words, the effects of volume fraction and diffusion permeability will be inseparably mixed. In contrast, when α does not vary in space, it can influence diffusion exclusively via the source term s/α and therefore acts merely as a concentration scaling factor. Expressed mathematically, Eq. 1 then simplifies to its traditional form

$$\frac{\partial c}{\partial t} = D_{\text{agar}} \nabla \cdot (\theta \cdot \nabla c) + \frac{s}{\alpha}.$$

When two parts of the environment meet at an interface where volume fraction or diffusion permeability abruptly change their values, we have to supplement Eq. 1 with conditions valid at the interface. They ensure that both the ECS concentration and the amount of substance transported across the interface remain continuous, preventing any unaccounted

for substance loss. Following the approach often employed in electromagnetic field theory, we construct a small cylindrical volume with one of its two circular faces above and the other below the interface. Gauss's theorem is then applied in the same way as before, while contracting the cylinder's height to a negligibly small value. It is immediately seen that the ECS concentrations just below the interface (subscript $-$) and just above it (subscript $+$) must satisfy conditions

$$c(\vec{r}_-, t) = c(\vec{r}_+, t) \\ \alpha(\vec{r}_-) \vec{n} \cdot [\theta(\vec{r}_-) \cdot \nabla c(\vec{r}_-, t)] = \alpha(\vec{r}_+) \vec{n} \cdot [\theta(\vec{r}_+) \cdot \nabla c(\vec{r}_+, t)], \quad (3)$$

where $\vec{n}$ is a unit vector perpendicular to the interface. Eq. 3 indicates once more that the effects of volume fraction and diffusion permeability in an inhomogeneous environment are intermingled.

In order to explain the experimental data as realistically as possible, we constructed a 3D numerical model based on Eqs. 1 and 3. At the RTI or RTP spatial resolutions, the SP layer boundaries can be considered planar and the source can be represented by a point placed at the origin of a cylindrical coordinate system (ρ, ϕ, z) . Individual layers were assumed to be homogeneous and isotropic. Layer interfaces were oriented perpendicularly to the axis of symmetry z . This arrangement eliminated all angular dependencies and therefore resulted in smaller computer memory allocation and faster computations. The diffusion equation was digitized along the two remaining spatial coordinates ρ, z and time t according to the Forward Time Centered Space (FTCS) finite difference scheme (Press et al., 1992). FTCS is a simple fully explicit scheme, which avoids difficulties associated with handling and inverting of large sparse matrices. On the other hand, the von Neumann stability criterion has to be satisfied, which necessitates smaller time steps.

Ideally the numerical model would be used to find the best parameter fit for every individual data set. However, computation of a single 150 s diffusion time course took several hours on a contemporary personal computer, which made such an approach impractical. Fortunately, both the experiments and the modeling revealed that within a wide range of plausible SP diffusion parameters, the diffusion curves could be fitted remarkably well under the (incorrect) assumption that the environment was perfectly homogeneous. It is important to realize that the "apparent" α^* and θ^* parameters fitted in this way cannot be used as estimates for the volume fraction or permeability in any part of the tissue. Indeed, they depend on electrode spacing, their typical values across the layer are clearly unrealistic, and the scalar α^* can even display directional dependence. To avoid any possible confusion, we decorate them with asterisks. Despite the loss of physical meaning, they can be treated simply as mathematical parameters characterizing the shapes of experimental curves. Their small standard deviations show that they are quite consistent and reproducible in this more modest role. We therefore obtained average values of α^* and θ^* from measurements across the SP layer and used them to construct a characteristic diffusion curve across the SP.

The characteristic diffusion curve across the SP was then used in an inverse problem of finding the α and θ of the SP layer. These are the only two parameters that had to be fitted for because the physical parameters of the other two layers and all the geometrical details including the SP thickness are known. We chose the downhill simplex algorithm (Press et al., 1992) for fitting, since it does not require computationally expensive evaluation of the derivatives. The iterations were stopped when the relative improvement of the mean squared difference between the modeled and the characteristic experimental diffusion curves became smaller than 0.01%.

We have implemented the FTCS numerical model and the inverse problem solution in the IDL language (ITT Visual Information Solutions, Boulder, CO), taking advantage of its built-in multi-threading capability when handling the time consuming convolution operators. Our software is available upon request under the GNU General Public License. The program was tested by comparing its output with known analytical solutions, after setting diffusion parameters of the SP layer identical to those of the adjacent SR and SO layers. The testing also found optimal values for spatial digitization steps and for overall dimensions of the simulation environment, which has to be made sufficiently large in order to avoid any significant influence from its outer edges.

After α and θ of the SP layer were found from measurements across the SP layer, we used these values for two additional sets of calculations. First, we verified that a diffusion curve predicted by the model in a direction parallel to the layer matched its corresponding experimental characteristic data set. Second, the model was used to demonstrate the influence of electrode spacing on diffusion curves both across the SP layer and parallel to it.

3 Results

3.1 ECS parameters in stratum radiatum and stratum oriens

We began by measuring the ECS parameters α and θ in SR and SO, the two layers adjacent to SP in the hippocampal CA1 region (Figure 1a). These layers are sufficiently thick for the RTI or RTP methods to quantify α and θ in a traditional way. We employed the RTI method in SR primarily for a comparison with already published results (McBain et al., 1990; Mazel et al., 1998; Hrabetova, 2005). Because the SO has not yet been characterized, we measured θ along all three coordinate axes to detect any possible anisotropy within this layer.

Figure 2a shows a representative RTI record from the SR. Table 1 summarizes mean values of the fitted volume fractions and permeabilities obtained from measurements along the x axis in 5 hippocampal slices from 5 animals. The non-specific clearance of TMA⁺ was $0.0157 \pm 0.0007 \text{ s}^{-1}$ (mean $\pm$ SD, $n = 15$).

In SO, we found with the RTP method that θ 's acquired along the x , y , and z axes were 0.444 ± 0.061 (mean $\pm$ SD, $n = 16$), 0.460 ± 0.072 ($n = 14$), and 0.441 ± 0.049 ($n = 17$), respectively. These results were obtained in 7 hippocampal slices from 5 animals. The mean values of θ did not differ significantly (ANOVA, $p > 0.05$) among the three spatial directions, indicating that the SO is isotropic. It is therefore possible to calculate α and θ in SO from the RTI experiments performed along a single spatial direction. An example of such record is shown in Figure 2b. Table 1 summarizes the mean values of α and θ obtained along the x axis in 3 hippocampal slices from 3 animals. The non-specific clearance was $0.0134 \pm 0.002 \text{ s}^{-1}$ ($n = 16$).

It remains to compare the diffusion properties of SR and SO. Statistical evaluation (Student's unpaired t test, $p > 0.05$) confirmed that the ECS volume fractions are not significantly different. Likewise, no significant difference was found in diffusion permeability. Because the diffusion properties of SR and SO were so similar, we pooled the values of α and θ obtained with RTI method in these two layers (Table 1). These grand averages were used to inform the numerical model.

3.2 Apparent ECS parameters obtained perpendicularly to stratum pyramidale

Figure 2c shows a representative RTI record taken across all three layers with electrodes 120 μm apart, placed symmetrically above and below the SP. The apparent values of α^* and θ^* characterize the shape of diffusion curves very well, and the fit quality is comparable to that

obtained in SR or SO. However, as explained in the Methods, they do not represent physically meaningful volume fraction or permeability of any of the three layers.

We acquired 15 diffusion curves across the SP in 5 slices from 5 animals and obtained $\alpha^* = 0.276 \pm 0.027$ (mean $\pm$ SD) and $\theta^* = 0.388 \pm 0.022$. The average values were used to construct a characteristic data set across SP, which represents a diffusion curve that would be obtained with a typical transport number of 0.35 and a typical iontophoretic current of 80 nA. Because the current and the transport number are mere scaling factors applied to the source, their exact values do not matter as long as the same values are used in the numerical model. However, the electrode spacing (120 μm) and the source duration (50 s) must be kept constant in all experiments and modeling. A small non-specific clearance factor was ignored.

To independently validate the numerical model based on diffusion experiments across the SP layer, we also acquired 6 diffusion curves in a direction parallel to the SP, with iontophoretic and ISM microelectrodes placed in the middle of SP. In 3 acquisitions, the microelectrodes were 90 μm apart, resulting in the apparent α^* of 0.197 ± 0.007 and θ^* of 0.453 ± 0.030 (Figure 2d, Table 2). The other 3 records were acquired with a microelectrode separation of 95 μm . The apparent α^* was 0.210 ± 0.005 and θ^* was 0.464 ± 0.001 .

Dependence of the apparent volume fraction on microelectrode spacing and orientation clearly indicates that the layered environment is not macroscopically homogeneous and isotropic. The average values of apparent parameters α^* and θ^* were again used to construct a characteristic data set, this time in a direction parallel to SP.

3.3 Numerical modeling

As described in the Methods, initial tests of the model found suitable numerical parameters that were used in all subsequent numerical experiments. Digitization intervals along the ρ and z coordinates were set to 2.0 μm , with 500 nodes along the ρ axis and 1000 nodes along the z axis. A complete environment therefore formed a cylinder of 2 mm diameter and 2 mm height. The source and detector microelectrodes were always placed to coincide with the nodes while both boundaries of the SP layer were positioned halfway between the nodes. The layer was assumed to be uniformly 50 μm thick. The diffusion coefficient D_{agar} was set to $1.24 \times 10^{-9} \text{ m}^2/\text{s}$ everywhere. This is a reference value for TMA⁺ at 34°C (Nicholson, 2001), checked for consistency in agarose gel before and after every experiment. The inverse problem calculations used the first 60 s but after the fitting was finished, diffusion curves were calculated with the obtained parameters for a full time interval 0 to 150 s matching the experiment.

The required number of iterations in the inverse problem algorithm depends heavily on the quality of the initial estimate. Because every iteration is time-consuming, we first ran the algorithm with very low resolution in order to obtain a good starting point for a more refined subsequent run. Specifically, this preliminary run used 10 $\times$ coarser spatial mesh than the final run, which relaxed the von Neumann stability criterion by a factor of 100 and accelerated the computation by a factor of 10,000, providing a starting point $\alpha = 0.136$ and $\theta = 0.309$ for the fine resolution run. This refined run required 43 iteration steps which took over 30 hours of computational time and yielded estimates of $\alpha = 0.127$ and $\theta = 0.327$ in the SP layer (Figure 3a and Table 1).

The estimates of diffusion parameters in the SP layer obtained above make the model of hippocampal CA1 region complete. We are now in a position to predict results of diffusion experiments performed with other source timings or electrode distances, or acquired along different directions. With geometric parameters adjusted to emulate diffusion experiment

inside the SP layer in a direction parallel to it, the model predicted apparent $\alpha^* = 0.192$ and $\theta^* = 0.459$ (Figure 3b and Table 2). This is in excellent agreement with the experiment and thus provides a useful validation of the model.

To demonstrate dependence of both apparent parameters α^* and θ^* on the electrode distance and orientation, a feature unique to an inhomogeneous environment, we simulated diffusion experiments along both directions and with electrode distances from 30 μm to 150 μm . The results are summarized in Table 2 and Figures 3c and 3d. In contrast to the intrinsic layer parameters, the apparent parameters depend strongly on electrode distance and direction. Such dependence gives an indication that the apparent parameters cannot be interpreted as physically meaningful volume fraction and diffusion permeability of the environment.

4 Discussion

The main result of this study is a detailed characterization of ECS properties in the CA1 region of hippocampus. We constructed a more realistic 3D model of its layered structure and extracted the ECS parameters of each individual layer. This would not otherwise be possible due to the small thickness of the SP in comparison with typical spatial resolution of available experimental methods.

Layered tissue structure, which is found in many other places in the nervous system including the retina, was shown to have important and somewhat counter-intuitive implications for diffusion in the ECS. The most striking feature is illustrated in Figures 3a and 3b: The molecules moving from the source to the detector diffuse in both the SP and the SR/SO environments. One might therefore assume that the diffusion curves, and by extension the apparent parameters extracted from them, would resemble an average taken from the SP environment alone (curves labeled “thick layer”) and the SR/SO alone (curves labeled “no layer”). Clearly, this is not the case. Perpendicularly to the layers (Figure 3a and Table 2), the diffusion curve and the corresponding apparent parameters have little in common with averaged curves or parameters of the individual layers. In particular, the apparent α^* across SP is higher than the volume fraction of any individual layer and both apparent parameters depend on the electrode distance. Similarly, the apparent θ^* in a direction parallel to the layers can be higher than any of the individual diffusion permeabilities, depending again on distance.

To see why this is so, let us consider the simplest possible case of diffusion across a thin layer where the ECS volume fraction is reduced but which is otherwise exactly the same as its surroundings. Given identical ECS concentrations at the source, the diffusion over small distances inside the layer will look identically to the diffusion outside because the diffusion permeabilities are the same. However, molecules crossing the layer from outside will encounter a sudden transition to lower ECS volume fraction, which will reflect some of them back (Figure 4). When their depleted numbers reach the other side, the molecules are released into a larger volume fraction, effectively reducing their concentration. The layer thus functions somewhat like a tennis training wall full of holes, randomly passing or reflecting the molecular balls thrown at it. If its presence was ignored, the much reduced concentration around the detector would be incorrectly interpreted to mean a very large ECS volume fraction.

One important conclusion of this study therefore is that ignoring inhomogeneities existing on a distance scale comparable to the spatial resolution may lead to incorrect interpretation of the underlying ECS parameters. Spatial resolution of the RTI and RTP methods is on the order of 100 μm , and similar resolution is achieved with the Integrative Optical Imaging method (Nicholson and Tao, 1993). However, typical resolution of the widely used diffusion

tensor imaging method (Basser et al., 1994) is only about 3 mm. If some macroscopic inhomogeneity is present on that distance scale, the extracted apparent parameters, such as the apparent diffusion tensor and various indices based on it, will lose the physical meaning valid only in a homogeneous environment. Without detailed 3D analysis, such parameters will be just as difficult to interpret as the apparent α^* and θ^* .

The hippocampal CA1 region plays an important role in learning and memory, at the same time being unusually susceptible to seizures and ischemia. It is therefore not surprising that several diffusion studies examined it in the past. The RTI method was used primarily in rat hippocampal slices (McBain et al., 1990; Perez-Pinzon et al., 1995; Hrabetova, 2005) and in a rat brain in vivo (Mazel et al., 1998). Although all four studies concluded that the CA1 hippocampal region differed from other parts of the brain, there was a significant variability in the reported values of α and θ . In the light of present study, a likely explanation is that apparent α^* and θ^* were obtained in some cases instead of α and θ , particularly when the SP layer was involved. These apparent quantities do not represent physical properties of the tissue and depend considerably on the exact microelectrode placement, distance, and orientation. Despite these difficulties, there is a qualitative agreement. For example, the first RTI study in rat hippocampal slices (McBain et al., 1990) found that the ECS is smallest in the SP and becomes further reduced when electrographic seizures are induced. They also pioneered the idea that regional variation of the ECS properties underlie the disposition of CA1 region to seizures and ischemia.

Three studies conducted experiments involving either SP alone or SP in combination with SO. The already mentioned publication of McBain et al. (1990) reported very low values of α and θ acquired inside the SP in a direction parallel to it. However, because spacing of the microelectrodes was variable (66–150 μm), it is not possible to make a quantitative comparison. Similarly, Perez-Pinzon et al. (1995) reported measurements in SP of rat hippocampal slices with variable microelectrode spacing (100–150 μm), performed probably in a direction parallel to SP. Finally, Mazel et al. (1998) measured in vivo in a hippocampal region of combined SP and SO layers. The uncertainties in electrode placement and distances (100–200 μm) again preclude quantitative comparison but it is interesting to note in their Figure 1 (Mazel et al., 1998) that these authors found a strong dependency of the apparent volume fraction on the spatial direction (α^* ranging from 0.12 to 0.29). This is a feature predicted by the layered model and expected in an inhomogeneous environment.

Present results from the SR layer are in a good agreement with our previous RTI studies (Hrabetova, 2005: isotropic layer, $\alpha = 0.24$ and $\theta = 0.47$; Hrabetova et al., 2009: $\alpha = 0.23$ and $\theta = 0.45$). They also agree with two multiphoton imaging studies that used a small fluorescent marker (Alexa Fluor 350, MW 349) in brain slices from rats (Zheng et al., 2008: $\theta = 0.48$) and mice (Scimemi et al., 2009: $\theta = 0.48$). The earliest RTI study (McBain et al., 1990) in rat hippocampal slices, held in an interface chamber, reported smaller volume fraction (0.13) and permeability (0.34) in SR. The in vivo study conducted by Mazel et al. (1998) found $\alpha = 0.22$, similar to our result, but argued for SR anisotropy due to large hindrance perpendicularly to the layers. As the authors point out, it is very difficult in an in vivo experiment to ascertain the microelectrode position along this direction more precisely than about 100 μm . It is therefore possible that higher hindrance along this axis reflected inclusion of the adjacent SP layer. Indeed, recent study from the same group (Anderova et al., 2011) reported isotropic diffusion in the SR of rat brain slices.

To the best of our knowledge, there are no previous reports of ECS parameters in the SO layer. We have found that it is isotropic and very similar to SR.

Our conclusion that the CA1 region is not homogeneous is in a good agreement with detailed study of resistivity conducted by Lopez-Aguado et al. (2001). They showed that the SP had much higher resistivity than the SR and SO, and correctly speculated that the SP may have lower ECS volume fraction, which would reduce pathways available for passing ionic currents. A direct quantitative comparison between the diffusion and resistivity studies is complicated because electric (ionic) currents are not strictly extracellular (Nicholson, 1965). However, it is likely that the observed increases of the SP resistivity in response to repetitive neuronal activation are caused by cellular swelling leading to further reduction of the ECS volume and permeability.

5 Conclusions

We have developed a diffusion analysis for an inhomogeneous layered environment and used it to model diffusion in the CA1 region. Quantitative results of this model indicated that the stratum pyramidale has significantly smaller ECS volume fraction and lower permeability than the tissue surrounding it, and acts as a formidable diffusion barrier.

Beyond the hippocampus, there are other important applications for diffusion analysis in layered brain regions, both under normal and pathological conditions. For example, a healthy retina is composed of six layers spanning approximately 150–200 μm , depending on the species. It is thought that melatonin produced by its photoreceptors diffuses throughout all layers and modulates dark adaptation of various retinal processes (Redburn and Mitchell, 1989; Wiechmann et al., 2004). Similarly, dopaminergic neurons are confined to a thin sheet at the edge of the inner plexiform layer (Witkovsky and Schütte, 1991), and their signals are also mediated by ECS diffusion (Witkovsky et al., 1993).

A model extended to many thin layers can also approximate an environment where the ECS parameters vary smoothly from one location to another, which is typical for regions affected by pathological events. The impacted brain volume is often surrounded by an inhomogeneous periphery with gradual transition into healthy tissue. The penumbra associated with focal ischemia is an obvious example (Paciaroni et al., 2009). Tumors represent an especially important case because they usually grow faster than their capillary network. This necessitates long-distance diffusion of nutrients and drugs (more than 100 μm) in a spatially variable ECS environment, with consequences for the treatment outcome (Minchinton and Tannock, 2006; Jain, 1990).

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Highlights

- Stratification of brain structures profoundly affects extracellular diffusion.
- A generalized diffusion equation for inhomogeneous environments is derived.
- Numerical solution for layered structures is implemented.
- Volume fractions and diffusion permeabilities of hippocampal layers are quantified.
- Stratum pyramidale is shown to act as a diffusion barrier.

Figure 1.

Experimental methods. **a**) Schematic anatomy of transverse rat brain slice modified with permission from Figure 32 of Paxinos and Watson (1997, copyright Elsevier), with magnified view of a hippocampal CA1 region. SR stands for stratum radiatum, SP for stratum pyramidale, and SO for stratum oriens. **b**) An experimental setup.

Tetramethylammonium ions, released from the source microelectrode by iontophoresis (RTI method) or by pressure pulse (RTP method) are detected by an ion-selective microelectrode positioned about 100 μm away. Amplified signal is digitized and stored on a computer. **c–d**) Sequence of diffusion curves obtained in brain slices and in agarose gel with the RTI and RTP methods. Lower parts of the graphs show the iontophoretic current I or applied pressure P . The labels show which variables are obtained. For example, the value of a diffusion coefficient D_{agar} is based on auxiliary experiments performed in agarose before the brain measurements, and checked again afterward to ensure consistency. See Methods for more details.

Figure 2.

Examples of typical RTI diffusion curves obtained with different placements and orientations of the source and detector microelectrodes in the CA1 region, as depicted on the inset cartoons. The experimental curves (gray solid lines) were fitted (black dashed lines) as described in the Methods. The fitting procedure resulted in estimates of the ECS parameters α and θ in the SR and SO (panels **a** and **b**). However, only the apparent parameters α^* and θ^* could be obtained from measurements across the SP ($\perp$, panel **c**), or parallel to it ($\parallel$, panel **d**). Numerical modeling was necessary to estimate the true structural values of α and θ in this thin layer. Timing of the iontophoretic source is denoted by a horizontal bar above the time axis.

Figure 3.

RTI experiments and numerical modeling of diffusion involving the SP. Following the procedure described in Methods, experiments were summarized by one characteristic curve for diffusion across the SP (solid line in panel **a**), and another curve for diffusion parallel to it (solid line in panel **b**). Variable r denotes distance between the source and the detector. **a**) Diffusion across the SP. Characteristic experimental curve is compared with three model situations. The points marked with squares were generated by a model which agreed best with the experiments. The ECS volume fraction and diffusion permeability of the SP which assured the best possible fit are presented in Table 1. Numerical model clearly agrees well with the experiments upon which it is based. The “Thick layer” curve corresponds to a SP of infinite thickness while the “No layer” curve models the opposite extreme of a missing SP. **b**) Diffusion parallel to the SP. All curves are analogous to those in panel **a**. However, the model was not informed by these experiments in any way. A very good agreement of model prediction with the characteristic experimental curve therefore serves to quantitatively validate the model. **c–d**) Dependence of the modeled diffusion behavior on diffusion distance and direction (panel **c**: perpendicularly to SP; panel **d**: parallel to it). The inhomogeneity caused by SP is manifested differently in different directions. Data point symbols show every 25-th time point generated by the model. The curves fitted through these data points correspond to apparent volume fractions and diffusion permeabilities shown in Table 2. Strong dependence of the apparent parameters on diffusion distance provides an excellent warning that the ECS environment is not homogeneous.

Figure 4.

A schematic illustration of the layered environment with SP acting as a diffusion barrier (panel **a**), compared with the homogeneous environment (panel **b**). Diffusion source is marked with a cross, the molecules are represented by small dots moving away from it along the concentration gradient, and the cellular elements are drawn as squares, larger inside the SP. See Discussion for details.

Table 1

Summarized ECS diffusion properties of the three layers in rat hippocampal CA1 region in vitro.

Layer	$\alpha \pm \text{SD}$	$\theta \pm \text{SD}$	λ	Measurements
SR	0.216 ± 0.027	0.444 ± 0.017	1.50	15
SO	0.220 ± 0.026	0.453 ± 0.033	1.49	16
SR + SO	0.218 ± 0.026	0.447 ± 0.027	1.50	31
SP	0.127 ± 0.005	0.327 ± 0.010	1.76	15

The SO + SR line reports an average of pooled measurements in stratum radiatum and stratum oriens. See the Methods and Results sections for a detailed explanation of stratum pyramidale parameters.

Table 2

Summary of RTI experiments and numerical simulations.

Distance [μm]	Direction	Fitted α^*	Fitted θ^*	λ^*
30	$\perp$	0.104	0.650	1.24
60	$\perp$	0.306	0.357	1.67
90	$\perp$	0.289	0.373	1.64
120	$\perp$	0.278	0.384	1.61
RTI $\rightarrow$ 120	$\perp$	0.276	0.388	1.61
150	$\perp$	0.270	0.391	1.60
30	$\parallel$	0.093	0.660	1.23
60	$\parallel$	0.151	0.525	1.38
90	$\parallel$	0.192	0.459	1.48
RTI $\rightarrow$ 90	$\parallel$	0.197	0.453	1.49
120	$\parallel$	0.211	0.435	1.52
150	$\parallel$	0.218	0.426	1.53

Results of the RTI experiments are typeset in bold. Numerical simulations used $\alpha(\text{SR}) = \alpha(\text{SO}) = 0.218$, $\theta(\text{SR}) = \theta(\text{SO}) = 0.447$, $\alpha(\text{SP}) = 0.127$, and $\theta(\text{SP}) = 0.327$.