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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 ($\alpha$) and diffusion permeability ($\theta$). 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.



## 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 require 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 μm. 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.

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Diffusion experiments quantify two macroscopic ECS parameters. One parameter is the ECS volume fraction $\alpha$, defined as a proportion of the tissue volume occupied by the ECS ($\alpha = V_{ECS}/V_{tissue}$). Another parameter, diffusion permeability $\theta$, quantifies how much the diffusion process in a complex ECS environment slows down in comparison to an obstacle-free medium. It is defined as $\theta = D_{ECS}/D_{agar}$, 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 $\lambda = 1/\sqrt{\theta}$ (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, $\alpha$ 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 $\theta$ 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 $\alpha$ 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 $\theta$ 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) (Fig. 1a). The SP layer is fairly thin, only about 50 μm (Turner et al., 1991; López-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 $\alpha$ and $\theta$ 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 (Fig. 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 superfused with ACSF at a flow rate of 2.0 mL/min delivered by peristaltic pump. The ACSF temperature was maintained at 34 ± 1 °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 UMPlanFl, 10×, numerical aperture 0.3) and infrared differential interference contrast optics for visualization of the specimen and the microelectrodes (Fig. 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 μm 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).