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Electroosmotic perfusion of tissue: sampling the extracellular space and quantitative assessment of membrane-bound enzyme activity in organotypic hippocampal slice cultures

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Abstract

This review covers recent advances in sampling fluid from the extracellular space of brain tissue by electroosmosis (EO). Two techniques, EO sampling with a single fused-silica capillary and EO push–pull perfusion, have been developed. These tools were used to investigate the function of membrane-bound enzymes with outward-facing active sites, or ectoenzymes, in modulating the activity of the neuropeptides leu-enkephalin and galanin in organotypic-hippocampal-slice cultures (OHSCs). In addition, the approach was used to determine the endogenous concentration of a thiol, cysteamine, in OHSCs. We have also investigated the degradation of coenzyme A in the extracellular space. The approach provides information on ectoenzyme activity, including Michaelis constants, in tissue, which, as far as we are aware, has not been done before. On the basis of computational evidence, EO push–pull perfusion can distinguish ectoenzyme activity with a ~100 μm spatial resolution, which is important for studies of enzyme kinetics in adjacent regions of the rat hippocampus.

Keywords

Organotypic hippocampal slice cultures; membrane-bound enzymes; enzyme activity; electroosmotic sampling; neuropeptides; thiols

Introduction

Obtaining samples of the extracellular space

Tools for measuring extracellular concentrations of chemicals in the brain have been around for more than half a century. MacIntosh and Oborin introduced the cortical cup in 1953 [1], Gaddum the push–pull cannula in 1961 [2], and Delgado the chemitrode [3] and the dialytrode [4] in 1962 and 1970, respectively [5]. Improvements in push–pull perfusion [6–10] were followed by microdialysis, the most widely used method for sampling the extracellular space [11]. These tools were motivated by the need to establish a relationship between behavior and regional chemistry of the brain and to monitor changes in brain chemistry in response to specific disease states. These early sampling methods were an advance, but they were not optimal when considering criteria including damage radius and degree, mechanical obstruction by tissue, spatial and temporal resolution, and solute recovery. Several new tools for probing neurochemistry, in particular low-flow push–pull perfusion, direct sampling, and work from our laboratory on electroosmotic (EO) sampling and EO push–pull perfusion, promise better spatial resolution, less damage, and the ability to make quantitative measurements of ectoenzyme activity (specific citations are found below).

In microdialysis, a membrane permeable to water and small molecules separates the brain from a fluid perfusing the luminal volume inside the membrane. The membrane enables transport of water and small molecules (the size depending on characteristics of the cylindrical membrane) across the membrane into the lumen. The luminal volume is connected to inlet and outlet ports. Solute exchange occurs by diffusion. The perfusion fluid's velocity affects the mass transport by its effect on the concentration gradient during diffusion [12], although with high-molecular-weight (MW)-cutoff membranes, flow through the membrane into the tissue can occur [13]. Recent improvements include Stenken's use of affinity-based recovery enhancement via molecular receptors including cyclodextrins (for nonspecific binding to low-MW molecules, for example YGGFL and YGGFM), antibody-immobilized beads (for binding to cytokines and endocrine hormones), heparin (for binding to human cytokines), and bovine-serum-albumin–heparin conjugates (for cytokine and tumor necrosis factor- α recovery) [14–16]. Membranes with larger MW cutoffs are available for detection of large molecules, for example some peptides and proteins [14, 15, 17, 18]. Microdialysis has also been coupled to flow segmentation to achieve temporal resolution as low as 15 seconds [19].

In recent years, the Shippey group revisited the push–pull cannula and developed a novel method called low-flow push–pull-perfusion sampling (LFPS). In this concentric design, flow is directed from outer tubing into the inner tubing, generating a low flow rate (10–50 nL min⁻¹) while reliably producing 70–80 % recovery in vitro. It also provides better spatial resolution than microdialysis. The Shippey group successfully used LFPS to quantify the basal level of glutamate in the rat striatum [20], determine the relative levels of amino acids in vitreous humor and the vitreoretinal interface of the eye [21], and study glutamate release in the lateral hypothalamus during feeding and drinking [22]. When coupled to capillary electrophoresis (CE), LFPS can be used to measure basal and K⁺-stimulated ascorbate levels

at the rat vitreoretinal interface [23]. The Kennedy group coupled LFPS to flow segmentation to achieve 7 s temporal resolution at 50 nL min⁻¹ flow and, after further miniaturizing the probe inlet, achieved 200 ms temporal resolution at 30 nL min⁻¹ flow [24]. Kennedy et al. also investigated the method of direct sampling. In this technique, a fused-silica capillary tube with 90 µm outer diameter (o.d.) was used for sampling extracellular levels of glutamate, aspartate, glycine, phosphoethanolamine, and γ-aminobutyric acid in rat striatum. It yielded flows of 1–50 nL min⁻¹, 90 s temporal resolution, and 500 times better spatial resolution than microdialysis [25].

We have developed sampling approaches based on EO flow in tissue that address some of the limitations of the foregoing. Specifically, we wanted a method that could be applied equally well to small tissue cultures and potentially in vivo, that had useful and controllable spatial resolution, and that could be used for determining endogenous compounds and measuring the biochemical fate of exogenously applied compounds while causing minimal damage. In particular, there is a dearth of methods designed to determine ectoenzyme activity in intact tissue. The EO methods described below are well-suited to this problem. In addition, EO flow is much less affected by the size of the fluid paths in a porous medium than pressure is. As a result, the fluid flow generated electroosmotically will be more homogenous (in space) than pressure flow. Before providing a background on electroosmotic flow and describing its use in sampling, it is appropriate first to review a few important facts about ectoenzymes.

Ectoenzymes

Ectoenzymes comprise a family of membrane-bound proteins whose catalytic domains face the extracellular space (ECS). It is a diverse family of enzymes, with substrates that include both nucleotides and peptides. Their function is usually believed to be clearing active species from the ECS [26]. However, more recent studies suggest that ectoenzymes are also capable of more subtle biomolecular modulation, as will be described below.

Receptors for nucleotides are expressed on nearly all cell types [27]. Ectoenzymes that cleave nucleotides in the ECS, called ectonucleotidases, were first discovered in the 1940s. Since then several families of these enzymes have been identified on the basis of spatial distribution and functional differences. Ectonucleotidases are involved in the salvage and/or hydrolysis of adenosine monophosphate (AMP) and adenosine triphosphate (ATP), thereby having a function in purinergic transmission and energy metabolism [27–35]. The importance of these enzymes is further illustrated by their functions in a variety of physiological processes, including angiogenesis, immune response, and neurogenesis [27]. In addition, a specific family of ectonucleotidase, CD38/157 (EC 3.2.2.5), has a function in NAD⁺ metabolism and chronic lymphocytic leukemia [36–39].

Similar to their nucleotide-hydrolyzing counterparts, ectopeptidases not only activate and inactivate [40] but in some cases can regulate the bioactivity of peptides in a variety of physiological processes, including growth, cell survival, and stress responses [41–45]. Dipeptidyl peptidase IV (DP-IV, DPP-IV, DPP-4, or CD26, EC 3.4.14.5) cleaves hormones that regulate insulin release in the gastrointestinal system and cleaves neuropeptide Y (NPY) into the fragment NPY(3–36), which is a selective agonist for the Y2 class of NPY receptors

[46–48]. Another ectopeptidase, membrane alanyl-aminopeptidase (aminopeptidase N, APN, CD13, or mAAP, EC 3.4.11.2), is a type II integral zinc-dependent metallopeptidase that hydrolyzes angiotensin III and other hormones and the neuropeptide family of enkephalins and endorphins [49–51]. As evidenced by these substrates, there is evolving support for the theory that APN is involved in the regulation of arterial blood pressure and pain [49, 51, 52]. Neutral endopeptidase (NEP, neprilysin, endopeptidase, enkephalinase, EC 3.4.24.11) [53] degrades toxic A β -amyloid peptides that are associated with Alzheimer's disease [54]. It also hydrolyzes enkephalins and neuropeptide Y, suggesting that it has functions in pain and in energy metabolism [46, 51, 55]. As a result of and as evidence for their importance, DPIV, APN, and NEP are all therapeutic targets for pharmacological treatment of specific disease states [40, 51, 56]. Additionally, with angiotensin-converting enzyme (ACE, EC 3.4.15.1) and disintegrin-metalloproteinases (EC 3.4.24.81), these three ectopeptidases are involved in tumor development and metastasis [57].

Recently, it was discovered that the activity of neurolysin (endopeptidase 24.16, microsomal endopeptidase, mitochondrial oligopeptidase, neurotensin-degrading enzyme, EC 3.4.24.16) is altered in stroke [58]. This endopeptidase is expressed in neurons and astrocytes. In neurons, but not astrocytes, approximately 10 % of the enzymatic activity is membrane-bound [59]. Wangler et al. [60] report that “membrane- anchoring mechanism (s)” are not understood. Clearly, analytical techniques with the objective of measuring membrane-bound (ecto) enzyme activity would be useful.

Conventionally, ectoenzyme activity in tissue or cell culture is determined by adding the substrate to medium containing whole cells or membrane fractions isolated after homogenization. Products in the supernatant can be quantified using a colorimetric method, radiochemical assay, or HPLC analysis after further treatment. The reaction rate and the ectoenzyme activity can then be estimated [50, 61–67]. It is also informative to examine changes in translational precursors (mRNAs) to determine changes in ectoenzyme synthesis, using reverse-transcription polymerase chain reaction (RT-PCR), northern blot, and in-situ hybridization coupled to immunohistochemistry [68–70]. We are not aware of methods other than ours for determining ectoenzyme levels in intact tissue.

EO-flow basics

Bulk fluid movement called electroosmosis results when an electric current is passed through electrolyte-filled channels with charged walls, ranging from fused-silica capillaries to soil. We have found that electroosmosis occurs in brain tissue [71, 72]. Electroosmosis is the result of the presence of an excess of mobile counterions near fixed surface charges. Because of electrostatic and entropic factors, counterions in the electrolyte solution form a double layer adjacent to the charged surface of walls (e.g. in a capillary). There may be strongly adsorbed counterions reducing the surface charge. A more diffuse layer extending some distance away contains the remainder of the counterions required for charge neutrality (Fig. 1). The diffuse layer is therefore charged and moves under the influence of an externally applied electric field parallel to the interface. There is an easily measured but difficult to define property of surfaces that support electroosmotic flow: the ζ -potential. There exists a shear plane somewhere within the double layer separating surface-bound

water (solvent) from water (solvent) moving under the electroosmotic force. The electrostatic potential at this shear plane with respect to a point far outside the double layer where the electrolyte is locally electroneutral is the ζ -potential. The application of an external electric field parallel to the wall surface induces movement of the diffuse layer, which, in the steady state, “carries” the rest of the macroscopically neutral, bulk solution with it via the diffusion of momentum. It is important to note that the system as a whole, the surface plus the surrounding electrolyte, must be electroneutral. The net surface charge density, defined as the fixed charge density minus adsorbed counterion density, is related to the ζ -potential. Equation 1 reveals that the ζ -potential can be determined experimentally by measuring the interstitial velocity, v_{eo} , of an electrolyte solution with a specific permittivity, ϵ_w , and dynamic viscosity, η , in a local field with magnitude E :

$$\zeta = - \frac{v_{eo}\eta}{\epsilon_w E} \quad (1)$$

It is convenient to express the electroosmotic flow (volume/time) in terms of the applied current when working with systems having a series of connected but independent conducting elements including an electrolyte-filled capillary and a tissue slice. This relationship is summarized in the following equation:

$$U_{eo} = - \frac{\epsilon_w \zeta}{\eta} \frac{i}{\sigma_{el}} = \mu_{eo} \frac{i}{\sigma_{el}} \quad (2)$$

where U_{eo} is the electroosmotic flow rate, i is the current, and σ_{el} is the bulk conductivity of the electrolyte solution in the absence of a porous medium [73]. The electroosmotic mobility, μ_{eo} , is implicitly defined in Eq. 2. We note that the physical properties of the porous medium, namely porosity and tortuosity, are accounted for in the derivation of Eq. 2 despite the fact that they are not explicitly seen in the equation. For example, a reduction in a medium’s porosity increases the electric field across the medium (at constant current), resulting in an increase in the electroosmotic velocity so that the flow rate is maintained.

Fused-silica capillaries have a negative ζ -potential. Our capillaries, conditioned with sodium-hydroxide solution, have a ζ -potential of -46.5 ± 1.2 mV (one standard deviation) at pH 7.4, which is consistent with the literature [74]. Therefore, electrolyte solutions can be driven through fused-silica capillaries by electroosmotic flow. We have found that rat organotypic-hippocampal-slice cultures (OHSCs) also have a significant ζ -potential [71, 72]. The tissue is a porous medium, a bed of nonconducting particles (cells) (under non-electroporating conditions [75]). The surfaces of these “particles” are functionalized with proteins, anionic carbohydrates, and phospholipids that act as stationary negative “wall” charges. The ζ -potential of OHSCs is -22.8 ± 0.8 mV [71, 72]. Thus, passing a current through this brain tissue is accompanied by electroosmotic flow.

It is worthwhile to compare fluid flow and solute transport in a porous space by electroosmosis to that by pressure. Darcy’s law relates a pressure gradient to the superficial fluid velocity, v_p , in a porous medium, by Eq. 3:

$$v_p = - \frac{\Delta P}{L} \frac{\kappa}{\eta} \quad (3)$$

The superficial velocity is the flow rate divided by the total (i.e., pores and obstructions) cross-sectional area of the porous medium. The analogous expression for electroosmotic velocity (superficial) is given by Eq. 4:

$$v_{eo} = \frac{\Delta V}{L} \frac{\varepsilon_w \zeta}{\eta} \quad (4)$$

In Eq. 3, the pressure difference, P , over distance, L , drives fluid through a medium with a hydraulic permeability κ , (in units of m^2). For a cylindrical open tube of radius a , κ is $a^2/8$. Substitution of this value for the hydraulic permeability of a cylindrical open tube into Eq. 3 leads to the familiar Hagen–Poiseuille law. Thus, the hydraulic permeability relates to the cross-sectional area of individual channels or interstitial spaces containing the fluid in a porous medium. The hydraulic permeability for gray matter in the brain is approximately 10^{-15} m^2 , but it is anisotropic and significantly larger for white matter: as much as three orders of magnitude larger along axonal tracts and two orders of magnitude larger across tracts [76, 77]. As a consequence, fluid flow induced by pressure in the brain travels most readily along white matter tracts and in perivascular spaces [78]. In addition, brain tissue can undergo deformation, meaning physical perturbation caused by pressure can lead to alteration of the tissue structure. This in turn has a significant effect on the hydraulic permeability [77].

Fluid flow driven purely by an electric field (current flowing in electrolyte solution) through porous media of uniform ζ -potential is not accompanied by a pressure drop. Thus, variability in the hydraulic permeability has little to no effect on the pattern of electroosmotic flow in a complex porous medium. Because pressure is not created, there is also no deformation of tissue. The situation is different when the flow or current passes through two regions with different ζ -potentials, however. Pressure gradients are created in such systems [79]. Figure 2 shows a pair of surface plots that illustrate this principle. Other than the ζ -potential, all properties of the adjacent porous media are identical for the two panels. The downward arrow shows the direction of the flow path. Because the fluid is virtually incompressible, the fluid flow rate must be the same in each of the three serial sections of the material. However, because of the ζ -potential differences in the domains (Fig. 2, right panel), flow rate in the top and bottom domains is faster than that in the middle domain. Thus, to satisfy the law of conservation of mass, a flow-equalizing intersegmental pressure results at the domain interfaces. In general, it is difficult to determine the flow rate (or the velocity) without simulations in cases with intersegmental pressure because the magnitude of the pressure gradient depends on many factors, including hydraulic permeability, electric field strength, and differences in ζ -potential and in the lengths of the individual segments. (Fig. 3)

The objective of both pressure and electroosmotic flow is the transport of solutes out of the sample to an instrument or sensor. The effectiveness of solute capture depends on the ratio of the advective transport to diffusive transport. This is embodied in the Péclet number:

$$P_e = \frac{va}{D} \quad (5)$$

Here, v is the velocity of the fluid, a is a characteristic distance, and D is the diffusion coefficient of the solute. Thus, for example, the probability that a molecule is captured in a sampling process based on fluid flow in the tissue is proportional to the fluid velocity and a characteristic distance over which the molecule must diffuse to escape the flow field, but inversely proportional to the diffusion coefficient of the solute. The characteristic distance (a) is related to the inner diameter (i.d.) of the sampling capillary.

EO perfusion and sampling with a single capillary

Investigations of physiological and molecular processes require appropriate in-vitro models that maintain both functional and anatomical integrity. We use organotypic-hippocampalslice culture (OHSC). OHSC has been widely used for studying brain function and disease, including neurogenesis [80–82], neurotoxicity and/or cell death [83–89], synaptic plasticity [80, 84], and neuroprotection [80, 86, 90, 91]. Preparations of OHSCs from 6–7-day-old (p6–7) rat pups were based on modifications of the original technique by Stoppini, and can be cultured for up to 2 months in vitro [92, 93].

The cultures grow on a porous “insert” membrane that rests on growth medium or buffer. The cultures obtain nutrients by capillary action through the membrane and oxygen from the 95 % air–5 % CO₂ environment in the incubator. Figure 4 shows how the peptide is drawn through the tissue electroosmotically in the single-probe approach. A fused-silica capillary is placed in contact with the tissue through a thin electrolyte layer. An electrical circuit is established through the electrolyte or tissue. The application of a voltage induces electroosmotic flow from the peptide-filled bath, through the tissue, and into the sampling capillary.

The flow rate of the fluid is not directly measurable. Furthermore, although the flow rate is proportional to current, the arrangement shown in Fig. 1 does not permit direct application of Eq. 2 because a single ζ -potential does not characterize the system. In cases where there is a change in ζ -potential along the fluid path, a flow-equalizing intersegmental pressure develops at the interfaces between domains of different ζ -potential, as seen in Fig. 2. As aforementioned, qualitatively, the ζ -potential in the capillary is more negative than that in the tissue, so the inherent electroosmotic velocity of the capillary is larger than that of the tissue. The tissue’s hydraulic permeability does not enable the capillary’s fluid to flow at its inherent electroosmotic velocity, because pulling fluid through the dense tissue requires (negative) pressure. In the final analysis, depending on the two ζ -potentials and the pressure–velocity relationships in the two regions of differing ζ -potential (e.g., Darcy’s law or the Hagen–Poiseuille equation), the overall velocity and flow rate is intermediate between the two extremes [73, 94].

The spatial resolution of the technique is, in theory, dictated mostly by the current path, the diameter of the capillary, and the diffusion coefficient of the solute. In the tissue (Fig. 4) the current density (current/area) decreases with the depth into the tissue. Current density is directly proportional to electric field, which dictates the electroosmotic velocity. Therefore, the electroosmotic velocity (distance/time) decreases away from the capillary tip. It is convenient to consider the Péclet number (Pe) defined above in Eq. 5. When Pe is greater than one, the deterministic velocity is dominant. When Pe is less than one, diffusion dominates. In the latter case, solute can escape the influence of the fluid flow. A dashed line in Fig. 4 shows a hypothetical Pe of unity. Note that the sampling volume is much larger than the diameter of the capillary lumen. The spatial resolution of the EO sampling is therefore good, but it cannot be assumed that it is as small as the capillary-lumen diameter.

Hydrolysis of leu-enkephalin by ectoenzymes in CA3

Enkephalins are opioid pentapeptides that are involved in pain and immune response [51, 50] among other things. Studies have revealed that an endogenous blocker of enkephalin-hydrolyzing ectopeptidases has potent therapeutic properties [95]. We used our EO sampling method to study ectopeptidase activity in the OHSC. Figure 4 shows that the substrate-peptide leu-enkephalin, YGGFL, was in the bath below the tissue. We also added a D-amino-acid peptide D-YDAGDFL to the bath to act as an internal standard (IS). To determine the flow rate experimentally, we measured the number of moles of IS collected from the bath below the tissue in a fixed time. The flow rate depends on the applied current. For typical conditions (application of 23–45 μA current, or 3000–6000 V m^{-1} field, across a 30 cm, 150 μm i.d. capillary) the flow is in the range of 60–150 nL min^{-1} .

Cleavage of the N-terminal tyrosine from enkephalins inactivates them [96]. Thus we developed a capillary chromatographic separation of the substrate peptide, the IS, GGFL, and an external standard in the injected fluid, GGFM. Using EO perfusion we pulled YGGFL-containing solution through the extracellular space of the CA3 region of OHSCs. GGFL was determined by capillary liquid chromatography with a copper-tartrate postcolumn reagent and electrochemical detection [97–99] (Fig. 5). Quantitative measurement of the GGFL peak enabled the determination of the rate of hydrolysis of the YGGFL to GGFL. Experiments with a variety of inhibitors enabled Xu et al. to determine that the ectopeptidase responsible for YGGFL hydrolysis to GGFL in the CA3 region is a bestatin-sensitive aminopeptidase, for example APN. Xu also determined the Michaelis constant, K_M , for the ectoenzyme hydrolyzing YGGFL. The value of K_M agreed with literature estimates for bestatin-sensitive aminopeptidase. As far as we are aware, this was the first measurement of variables for enzyme kinetics in tissue. Tissue measurements, although more difficult, are preferred. Ectoenzymes solubilized by surfactants and the same enzymes residing in membranes may have different activities [100]. The physical structure of the extracellular space and the existence of other enzymes in the in-vivo environment more closely reflect the accessibility and reaction rate of the substrate with the ectoenzyme in vivo.

Catabolism of coenzyme A by ectoenzymes

Cytosolic biosynthesis of coenzyme A (CoA) is accomplished through the action of multiple cytosolic enzymes in five well-established steps [101–104]. Cysteine, pantothenic acid, and ATP are the three basic substrates for this biosynthesis. The degradation of CoA, however, is not as well understood [101–103, 105]. Stipanuk et al. proposed an integrated mechanism of cysteine and CoA metabolic pathways [105]. Interestingly, an ectoenzyme is involved in the degradation (Fig. 6): the degradation of pantetheine (PSH) to pantothenic acid and cysteamine (CSH), the last step in the CoA-degradation pathway, is catalyzed by the ectoenzyme pantetheine hydrolase (EC 3.5.1.92) [101, 103, 105].

To study this and other problems involving extracellular processing of thiols, we built an “all electric” microfluidic system for EO sampling coupled to a fluorogenic reaction, electrophoretic separation, and laser-induced-fluorescence detection [106–108]. As illustrated in Fig. 7, the tissue is situated as in Fig. 4. Reservoirs R1 and R3 are the main drivers of fluid flow. Reservoir R4 sinks most of the current from the sample stream coming from the culture dish. Reservoir R2 is used for gated injection. Perfusate from the culture is transported to the chip and thiols are derivatized using a fluorogenic maleimide reagent. Derivatized thiols are injected and separated by electrophoresis. Reservoir R4 serves as an isolator for the sampling and derivatizing steps. This reservoir is necessary for controlling the electric field across the sampling capillary without greatly affecting the following on-chip currents and flow rates. Moreover, it expands the usable potential range that can be applied to the sampling capillary and provides the option of splitting the sample flow to R4.

Coupling EO sampling to an EO-driven microfluidic system posed some challenges. One was the high conductivity of biological samples. Buffers used in microfluidic analysis typically have a much lower conductivity ($\sim 0.14 \text{ S m}^{-1}$ for 20 mmol L^{-1} Tris-HCl at pH 7.5) than the buffer used to perfuse the tissue (1.54 S m^{-1} for artificial cerebrospinal fluid (ACSF)). ACSF was necessary for maintaining osmolarity and simulating an in-vivo environment for OHSCs. Other than the well-known problems resulting from high conductivity, namely Joule heating, the formation of gas bubbles, and rapid electrolysis of buffer, high-conductivity samples can cause gated injection to fail. A direct solution was to find a low-conductivity substitute for normal ACSF. We replaced the major contributor to high conductivity in ACSF, NaCl, with the dipeptide GG, which effectively reduced the conductivity of the original buffer to 0.96 S m^{-1} . Propidium-iodide cell-death assays revealed that the GG-modified ACSF has minimal effects on the tissue in the sampling time scale. Furthermore, to prevent the failure of the gated injection caused by the mismatch of conductivity between the sample stream coming from the reaction channel and the running buffer from the gated reservoir, the running buffer was augmented with NaCl to a conductivity of 0.42 S m^{-1} . Using this set-up we successfully measured the basal concentrations of cysteamine, homocysteine, and cysteine in the extracellular space of the OHSCs (Fig. 8), which were $10.6 \pm 1.0 \text{ nmol L}^{-1}$, $0.18 \pm 0.01 \text{ } \mu\text{mol L}^{-1}$, and $11.1 \pm 1.2 \text{ } \mu\text{mol L}^{-1}$, respectively [107]. It is important to point out that GG is a substrate for γ -glutamyl transpeptidase (γ -GT), another ectoenzyme of interest in the regulation thiols (specifically in the regulation of glutathione, GSH), and is consequently a poor choice as a buffer

component for studies involving that specific enzyme and determination of extracellular GSH concentrations.

For measurements of substrate processing in the extracellular space, the substrate of the enzyme reaction or the drug of interest is incorporated into the culture dish. The tissue absorbs the fluid at the beginning of the experiment. On application of the electric field, the compounds of interest pass through the extracellular space of OHSCs with the sampling buffer, driven by the electroosmotic flow in the extracellular space. Each experiment results in a series of electropherograms in time. The signals from components of the sample (i.e., peaks in the electropherograms) reach a steady state.

On the basis of this idea, we obtained the apparent Michaelis constant ($16 \pm 4 \mu\text{mol L}^{-1}$) and maximum reaction rate ($7.1 \pm 0.5 \text{ nmol L}^{-1} \text{ s}^{-1}$) for the sequential degradation of CoA in the extracellular space of OHSCs. Using this method, we also successfully correlated the effectiveness and toxicity of two drug compounds to the distinct differences in their conversion rates to the active molecule, cysteamine. The disulfides cystamine and pantethine, the two drug compounds that treat cystinosis, yielded conversion rates of $91 \pm 4\%$ and $0.01\text{--}0.03\%$, respectively. The tissue-based measurements provide a direct way to monitor and evaluate drug metabolism in tissue and are potentially valuable for facilitating drug discovery.

It is important to understand that extracellular concentrations of solutes are not at equilibrium but at steady-state. The basal thiol concentrations in the tissue were determined by standard addition. This procedure is typically used in analytical determinations where so-called “matrix effects” occur. For the standard-additions approach to work, the interference must be proportional to the signal. For example, a matrix component may reduce a compound’s fluorescence by 10 %, e.g., dynamic quenching by oxygen. In such a case, standard additions are effective at providing a quantitative determination despite the interference. We note that first-order reactions occurring over a fixed time period also have an effect that is proportional to the ambient concentration of a compound. Thus, to the degree that the reactions, including uptake by cells, are first order, the standard-additions procedure will work for measurements of steady-state concentrations of solutes in the extracellular space of tissues.

Two probes: EO push–pull perfusion

Several neuropeptides have protective behavior in the hippocampus, a region highly susceptible to excitotoxic and inflammatory damage [109–113]. One such peptide, galanin (GWTLN SAGYL LGPHA IDNHR SFSDK HGLT-NH₂), reduces glutamatergic synaptic transmissions and, as a result, protects neurons against excitotoxic conditions that result from anoxia [114]. It also binds to galanin receptors that inhibit the production of pro-inflammatory cytokines including interleukin 1 (IL-1) and tumor necrosis factor- α (TNF- α) [115]. We are generally interested in how neuropeptides including enkephalins and galanin promote the survival of hippocampal neurons after an insult, for example oxygen–glucose deprivation (OGD), an experimental model for stroke [82]. The susceptibility of neurons to injury after OGD varies among regions within the hippocampus. In the rat and mouse, there

are significant anatomical and functional differences seen on the $\sim 100\ \mu\text{m}$ distance scale or smaller. To perform experiments on intact OHSCs that examine specific regions, better spatial resolution than that shown in Fig. 4 is required. Thus we developed a method, EO push–pull perfusion (EOPPP), using a pair of fused-silica capillary probes. Figure 9 illustrates the concept. The sampling capillary (analogous to a “pull” in push–pull; labeled “collect products” in Fig. 9) is positioned as in Fig. 4. However, the source (“push”) is a capillary pulled to approximately $20\ \mu\text{m}$ in diameter at the tip (labeled “peptide” in Fig. 9). Fluorescence-microscopy images of an EOPPP experiment performed with a Texas- Red-labeled 3 kDa dextran (TR3) in the push capillary reveal the trajectory of the probe molecule from the source tip to the collection capillary. A plume of dye is evident after 30 s. The volume of tissue interrogated is thus the region between the probes, which has been confirmed by simulation.

Using COMSOL Multiphysics (V4.3a), we determined that the flow-rate-to-current ratio for EOPPP is $1.51\ \text{nL min}^{-1}\ \mu\text{A}^{-1}$ in the sampling capillary. For the range of currents that we applied in our sampling experiments ($7\text{--}16\ \mu\text{A}$), this would translate to a flow-rate range of $10\text{--}25\ \text{nL min}^{-1}$. From the simulations, we have also determined that there is flow from the underlying buffer solution during sampling. This suggests that some dilution occurs as a result of this drawing of buffer from the solution underneath. Comparing the volume of internal standard (D-YAGFL) collected experimentally to that predicted by numerical calculations (COMSOL Multiphysics), we calculated a sampling efficiency in tissue of 20 % at the $16\ \mu\text{A}$ current applied. Here, we define sampling efficiency as the ratio of moles collected to moles delivered into the tissue. The sampling efficiency increases with current. In addition, the sampling efficiency was higher in tissue than in a gel (25 % (w/w) acrylic acid) with comparable ζ -potential [73, 116]. The increase in sampling efficiency with increasing current and the reduction in sampling efficiency in the gel can both be explained qualitatively using the Péclet number. In the former case, increasing the current increases the velocity while the diffusion coefficient and length scale remain unchanged, thereby increasing Pe and the dominance of advective over diffusive flux. In the latter case, the reduced tortuosity of the medium increases the diffusion coefficient and the increased porosity enables a greater diffusive flux away from the sampling path, reducing Pe .

We have applied this sampling technique to the quantitative determination of ectopeptidase activity acting on the 29-amino-acid neuropeptide galanin. Our sampling studies in the CA1 and CA3 of OHSCs reveal significant spatial differences in collection of galanin and galanin hydrolysis products, as revealed by capillary LC followed by offline MALDI-TOF. At high currents, the probability of having both short and long galanin fragments with intact carboxy termini (suggesting activity of an aminopeptidase) was higher in the CA3 ($p < 0.001$) than in the CA1. At low currents, the same trend was observed for short galanin fragments but not for long fragments (short: $p = 0.032$; long: $p = 0.503$). It is well known that the CA1 is more susceptible to ischemic stress than the CA3 [87, 89]. Our results suggest that the function of galanin, and the ectoenzyme activity that modulates this peptide, may also differ in two regions. This is the subject of our ongoing studies [73].

EO and damage

Measurements of extracellular processes can only be performed accurately in intact tissue. They are therefore among the most demanding and informative measurements that can be made. However, most if not all measurements in tissue are somewhat invasive. Measurements, including the ones presented here, that use probes, sensors, electrodes, etc. must be performed with an understanding of the potential effect of the probe itself or of the fluid flow initiated in the process on the measurement itself. For our work, a concern is the effect of the electric current or field on the tissue. From earlier work on electroporation [75, 117–120] we know that an electric field of approximately $6.7 \times 10^3 \text{ V m}^{-1}$ is required to electroporate a cell of typical dimensions, approximately 20–30 μm in diameter. This is in line with the observation that a cell can be electroporated if it experiences a transmembrane potential difference of approximately 200 mV. When the field diverges, as it does in our electroosmotic approaches, the situation is more complex. The cells closest to the probe(s) will experience the highest fields.

In practice, we have investigated the effect of single [121] and two-probe [122] experiments on cell death. These investigations involved changing the capillary dimensions and positions and the current or field to find conditions that were acceptable. Our determination of cell death was on the basis of propidium-iodide fluorescence 18–24 h after the procedure. The fluorescence measurements were sensitive to loss of any type of cell, neuron, astrocyte, or microglial cell. Unfortunately, the rules of thumb developed for the two procedures were not the same. Nonetheless, in both cases there are conditions that give rise to healthy flow rates and to minimum damage of less than 10 % cell death, compared with fully dead tissues. It is worth pointing out that our typical measurements span a long time, five or ten minutes. If in the process of acquiring a sample from the extracellular space a cell membrane was compromised, its picoliter contents would be collected but quite diluted thus having little effect on the measurement. It is possible that enzymes produced by cellular damage could affect results because the substrate peptide and the released enzyme would co-exist in the sampling capillary. We have looked for this and not found any degradation in the capillary [94]. In addition, in studies on galanin [73] we did not see significant cleavage at the carboxy side of proline, the favored cleavage site of one of the most abundant intracellular peptidases, prolyl endopeptidase (EC 3.4.21.26). The measurements of ectopeptidase activity are thus likely to reflect the natural processes within the tissue culture.

Conclusions

Our research group has developed two new tissue-perfusion methods based on electroosmotic flow under the influence of an external electric field. As far as we are aware, before our work published in 2010 there were no reports of tissue-based ectoenzyme kinetic measurements based on any method. Our methods result in minimal to no damage to the tissues themselves. Measuring kinetic variables of the ectoenzyme in tissue cultures can provide metabolic information more relevant to the biological organism than that obtained from currently practiced in-vitro experiments, in which homogenization results in loss of any spatial and temporal information.

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Fig. 1.
Schematic diagram illustrating the distribution of ions in the electrical double layer near a charged wall that leads to electroosmosis when a field is applied parallel to the surface

Surface Plot: Pressure (Pa)

Fig. 2.

Pressure created by variation of ζ -potential along the direction of the current flow. A field of 1000 V m^{-1} was impressed across the vertical dimension. ζ -potential values are indicated in their respective domains. Porous properties are the same for all domains and are defined as follows: porosity (ϵ)=0.4; tortuosity (λ)=1.4; conductivity (σ)= 1.43 S m^{-1} ; density (ρ)= $1 \times 10^3 \text{ kg m}^{-3}$; permeability (κ , calculated using the Kozeny–Carman equation for a packed bed of $10 \text{ }\mu\text{m}$ particles)= $5 \times 10^{-14} \text{ m}^2$; dynamic viscosity (η)= $8.9 \times 10^{-4} \text{ Pa s}$. The pressure at the top and bottom boundaries is set to the arbitrary value of zero so that the net pressure drop applied across each 0.8 mm-thick object is zero. Calculations were performed with COMSOL Multiphysics v4.4

Fig. 3.
Bright field image of organotypic-hippocampal-slice culture (4×0.16 NA objective, IX-71 inverted microscope, Olympus, Melville, NY).

Fig. 4.

Schematic portrayal of the single-probe system. An electrolyte-filled fused-silica capillary is placed perpendicular to the tissue culture and separated from it by a thin, liquid layer (25–50 μm). An electrode and the distal end of the capillary are placed in electrolyte. The other electrode is placed in the HBSS. Peptide substrate and a D-amino-acid peptide are dissolved in the HBSS. The approximately-semicircular lines indicate isopotentials

Fig. 5.

Five-minute sampling through CA3 at 5000 V m^{-1} ($38\text{ }\mu\text{A}$ current). The bath solution is HBSS with $150\text{ }\mu\text{mol L}^{-1}$ YGGFL and IS with or without inhibitors as follows: Experiment **A**, no inhibitors; **B**, includes inhibitors ($\mu\text{mol L}^{-1}$): thiorphan (15), GEMSA (210), captopril (25); **C**, as **B** plus puromycin (20); **D**, as **C** plus bestatin (100). Reprinted with permission from Xu et al. (2010) Electroosmotic Sampling. Application to Determination of Ectopeptidase Activity in Organotypic Hippocampal Slice Cultures. *Analytical Chemistry* 82 (15):6377–6383. Copyright 2010 American Chemical Society

Fig. 6.

Proposed biodegradation pathways of CoA. CoA is depicted at the top. Enzymatic hydrolysis occurs at the bonds indicated by *green dashed lines*. Note that potential path *B* involves an ectonucleotide pyrophosphatase. Path *C* also involves an ectoenzyme, pantetheinase. Reprinted with permission from Wu et al. (2013) Integrated Electroosmotic Perfusion of Tissue with Online Microfluidic Analysis to Track the Metabolism of Cystamine, Pantethine, and Coenzyme A. *Analytical Chemistry* 85 (24):12020–12027. Copyright 2013 American Chemical Society Electroosmotic perfusion of tissue

Fig. 7.

Integration of the electroosmotic sampling with online microfluidic analysis. The fused-silica capillary is 11 cm (length) × 50 μm (i.d.), 360 μm (o.d.). Adapted with permission from Wu et al. (2013) An in Situ Measurement of Extracellular Cysteamine, Homocysteine, and Cysteine Concentrations in Organotypic Hippocampal Slice Cultures by Integration of Electroosmotic Sampling and Microfluidic Analysis. *Analytical Chemistry* 85 (6):3095–3103. Copyright 2013 American Chemical Society

Fig. 8.

Detection of amino thiols in the extracellular fluid of OHSCs. The culture cell dish contains (red line) GG-modified aCSF only; or (black line) GG-modified aCSF with added cysteamine. At the beginning of the experiment, R1 contains $12.7 \mu\text{mol L}^{-1}$ ThioGlo-1 in a 20 mmol L^{-1} Tris-HCl buffer (pH 7.5); R2 and R3 contain 40 mmol L^{-1} Bis-Tris propane buffer with 15 mmol L^{-1} NaCl (pH 8.50); R4 and the sampling capillary contain 20 mmol L^{-1} Tris-HCl augmented with 60 mmol L^{-1} NaCl (pH 7.5). +3000 V, +300 V, and +4500 V are applied at the cell dish, R1, and R3, respectively. R2 is switched between ground (24.5 s)

and floating (0.5 s); R4 is connected to ground. There is a six-minute presampling step before each experiment, during which +3000 V is applied to the culture dish and R4 is grounded. All other reservoirs are floating. Electroosmotic sampling is performed in the CA3 region of the OHSCs. Adapted with permission from Wu et al. (2013) An in Situ Measurement of Extracellular Cysteamine, Homocysteine, and Cysteine Concentrations in Organotypic Hippocampal Slice Cultures by Integration of Electroosmotic Sampling and Microfluidic Analysis. *Analytical Chemistry* 85 (6):3095–3103. Copyright 2013 American Chemical Society Electroosmotic perfusion of tissue

Fig. 9.

Left: Schematic diagram of EOPPP. A continuous voltage is applied across the push capillary (labeled as “peptide”), the tissue, and the pull capillary (labeled as “collect products”). The resulting field drives electroosmotic flow of the peptide solution through the system from the push to the pull capillary. *Right:* Image of sampling scheme taken from below the tissue using an inverted microscope. The tip of the push probe in the tissue is indicated with an arrow. The tip of the sampling capillary (perpendicular to the tissue) can be seen as a dark circle outlined by a halo in all three micrographs (at $t=0$, 10, and 30 s, *left-to-right*). The flow of the dye from the push to the pull capillary can be seen as the increasing fluorescence of the lumen. The push tip is approximately $60\ \mu\text{m}$ below the pull capillary, which has an i.d. of $75\ \mu\text{m}$