In Vivo Measurements of Neurotransmitters by Microdialysis Sampling

With microdialysis, neuroscientists can interpret chemical changes in the brain caused by pharmacological and sensory inputs and gain new insights into a wide range of brain functions.

The brain contains a vast network of neurons that connect with each other at specialized junctions called synapses. A synapse consists of a presynaptic terminal (the "sending" neuron) and a postsynaptic bouton (the "receiving" neuron) that are separated by a gap of 5–50 nm (Figure 1). Chemicals released into this synaptic gap interact with receptors on the postsynaptic neuron. This leads to intracellular changes in the postsynaptic neuron—for example, an altered membrane potential or gene expression. The chemical signal is terminated by transporter proteins that transfer transmitter molecules across the membrane to the intracellular space (a process known as "reuptake") or enzymes that degrade the transmitter in the vicinity of the synapse (Figure 1). This classical view of neurotransmission might be considered point-to-point or "wired" communication because neurons communicate only with neurons to which they are specifically connected. In addition, neurotransmitters can activate receptors at more distant sites either by escaping the synapse or by being directly released into extrasynaptic space. This longer-range communication has been called "volume" transmission (*1*, *S1*; S references can be found in Supporting Information).

All brain functions, from controlling movement to emotions, involve these two forms of chemical communication. Analytical chemistry has an important role to play in developing our understanding of the brain by providing tools for identification and measurement of the many chemicals involved in neurotransmission.

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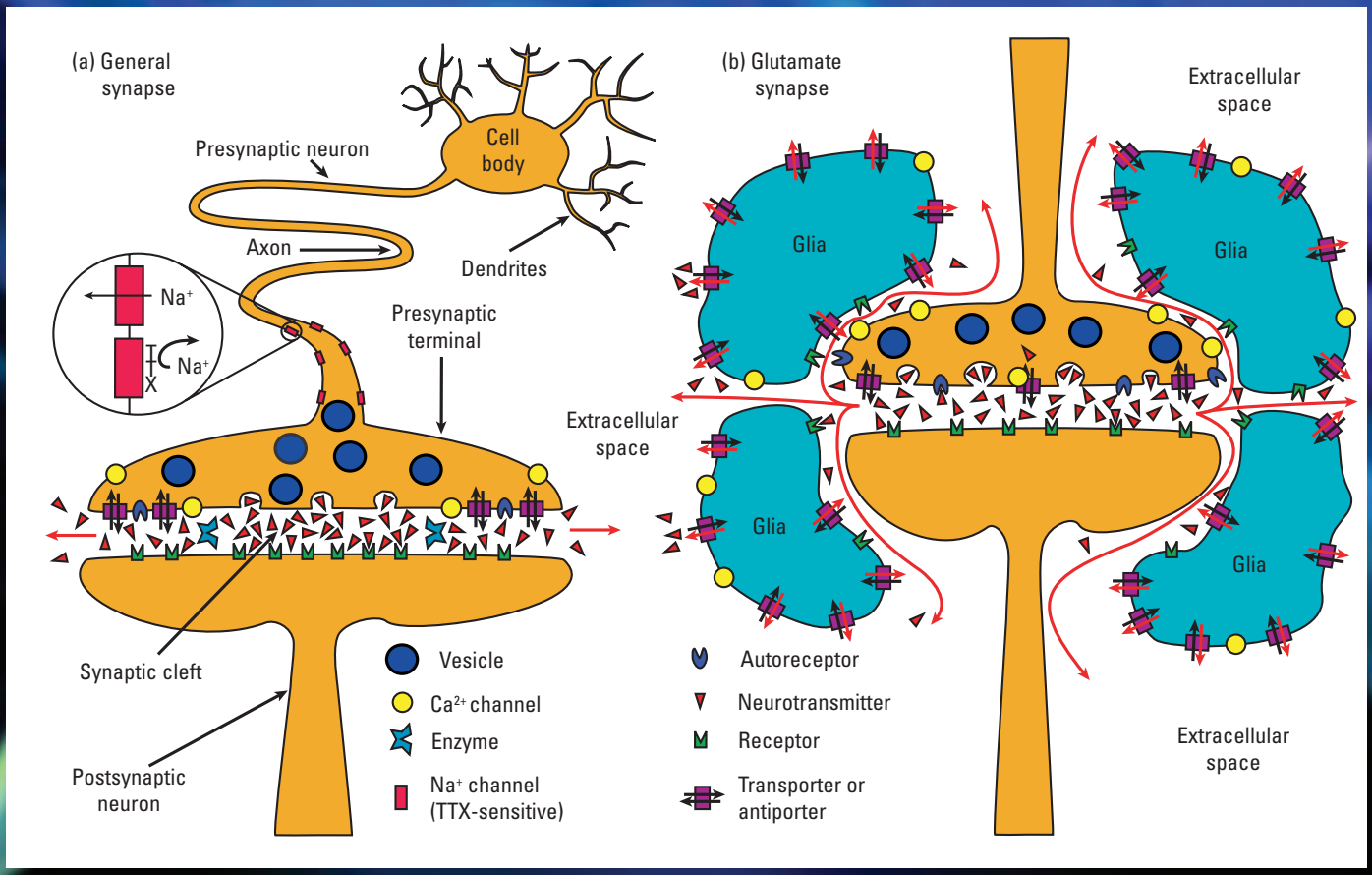


FIGURE 1. Neurotransmission at synapses.

(a) Depiction of generalized presynaptic neuron and postsynaptic bouton. Neuronal cells are composed of dendrites, soma, and axons. Action potentials that stimulate neurotransmitter release propagate through axons via Na^+ transport through ion channels. These channels can be blocked by the puffer-fish poison TTX (expanded view). Action potentials that reach the presynaptic terminal stimulate influx of Ca^{2+} from extracellular space, triggering the release of transmitter from vesicles by exocytosis. Neurotransmitter release can be blocked by TTX or by removal of Ca^{2+} from the extracellular media. These effects have been key tests for ascertaining the source of chemicals in dialysate. Released neurotransmitter can bind and activate receptors on the postsynaptic neuron for intercellular communication. Activation of receptors on the same neuron (autoreceptor) typically provides negative feedback on further release. Signaling is terminated by reuptake into the presynaptic neuron or by enzymatic degradation. Both point-to-point signaling and volume signaling (not shown) can occur. (b) Glutamatergic synapses are surrounded by glial cells that can take up and release glutamate by different mechanisms. (Release is indicated by red arrows through antiporter or transporter proteins in the glial cells.) Thus, the glial cells can contribute glutamate to the extracellular space. This additional source complicates the interpretation of glutamate concentration and concentration changes measured by microdialysis, which samples the extrasynaptic space.

In vivo measurements

In vivo measurements of neurotransmitters are invaluable because they allow researchers to investigate how the intact network regulates neurotransmission, how systemic drug intake affects specific brain regions, and how behavior correlates to brain chemistry. Another important benefit of in vivo measurements is that fewer animals are needed to conduct a study. For pharmacokinetic studies, it has been estimated that a >10-fold reduction in animal usage can be achieved. This is because in vivo methods allow controls and all time points to be obained with a single animal, whereas measurements of analyte concentrations in tissues require one animal for every time point and separate control animals (*2*). Although clearly valuable, in vivo measurements of brain chemicals are complicated by many factors, including the array of neuroactive compounds (>200 chemicals have been identified as neurotransmitters or neuromodulators); the heterogeneity of tissue; and the presence of other cell types, such as glial cells, that also may release and take up neurotransmitters.

In vivo chemical measurements can be made by noninvasive methods, such as positron emission tomography (PET), or by invasive methods, such as sensors or microdialysis sampling, that involve inserting probes into brain tissue. Although PET has the enormous advantage of being noninvasive, it is expensive, not well suited for small-animal studies, and limited to just a few neurotransmitters. Furthermore, it has modest temporal (<10 s) and spatial (<1 cm^3) resolution (*3*). For these reasons, probe techniques remain in high demand.

Microsensors have been refined to the point of achieving high temporal and spatial resolution (10 ms and tens of cubic micrometers, respectively, in the best cases). On the other hand, they have several limitations. Such sensors are available for just a few neurotransmitters. Measurements of basal concentrations remain problematic. The selectivity of the sensors can be difficult to verify. Finally, the high spatial and temporal resolution can be achieved for only a few transmitters; the resolution of many sensors is 10–1000-fold lower.

In contrast, microdialysis generally provides worse temporal and spatial resolution (600 s and ~0.1 mm^3, respectively, are typical) than a sensor, but it has other features that complement the sensor and PET measurements. As a sampling tool, microdialysis can be coupled to various analytical techniques—such as HPLC, CE, and enzyme assays—allowing virtually any neurotransmitter

or drug to be measured with high selectivity and sensitivity. In many cases, multiple analytes can be detected in one sample; this facilitates studies of neurotransmitter interactions. Because samples can be collected over time, microdialysis can be used to obtain both basal concentrations and dynamic information on brain chemistry. Over the past few years, the practice of coupling microdialysis to sensitive analytical techniques has dramatically improved the temporal resolution to just a few seconds. Finally, this method is relatively inexpensive and easy to use.

Microdialysis has become enormously popular because of many of the features mentioned earlier; >9000 papers that use this technique have been published. Perhaps the most important reason is this: With this method, neuroscientists have been able to consistently interpret the chemical changes that are observed in response to pharmacological and sensory inputs and to derive new insights into a wide range of brain functions (including sleep, addiction, sex drive, and cognition) and pathologies (such as ischemia).

For example, the role of adenosine in mediating sleep was further elucidated using microdialysis techniques, which revealed that extracellular concentrations of adenosine in the brain increased with longer periods of wakefulness and recovered during sleep. Impressively, sleep could be induced by increasing adenosine concentration in the vicinity of the dialysis probes. These results provided evidence that adenosine plays a causative role in inducing sleep (*4*). In another example, microdialysis was used to discover that glutamate is released to toxic levels during ischemia and is an important contributor to the brain damage associated with a stroke (*5*). A new treatment for schizophrenia has been proposed on the basis of observations that glutamate concentrations increased in an animal model (*6*). This observation led to the hypothesis, which was verified, that glutamate autoreceptor agonists might reduce glutamate levels and decrease schizophrenia-like symptoms. Microdialysis has also made significant contributions as a diagnostic tool in the areas of human neurosurgery, neurotrauma, and clinical studies (*7*, *8*, *S2–S16*, *S61–S64*).

Microdialysis sampling

A microdialysis probe consists of two pieces of microbore tubing sheathed in a semipermeable membrane that is plugged at one end (Figure 2). When the probe is inserted into a sample, molecules that fall below the molecular-weight cutoff of the membrane will diffuse across the membrane according to their concentration gradients. Perfusion of the interior of the membrane, typically performed at 0.1–3.0 μL/min, generates a stream of dialysate that can be analyzed for compounds of interest. For intracranial measurements, the interior of the probe is perfused with artificial cerebrospinal fluid to minimize the effect of sampling on the ionic environment of the brain extracellular fluid. Constant flow through the probe creates a concentration gradient between fluid in the probe lumen (dialysate) and fluid in the extracellular space. This gradient results in diffusion of analytes from the extracellular fluid, through the dialysis membrane, and into the dialysate. This principle can also be utilized to deliver compounds from the probe into extracellular space ("reverse dialysis"), which enables localized delivery of drugs.

When a microdialysis probe (or any type of sensor) is inserted into the brain, it resides in the extracellular space, which, for the purpose of this discussion, is considered a compartment that is distinct from the synaptic gap. The extracellular space is a tortuous network that occupies ~20% of the brain volume (*9*). This space is a conduit for neurotransmitters, metabolites, and drugs moving between neurons, glia, and the bloodstream; therefore, it is rich with information about the function of cells in the vicinity. Neurotransmitter concentration in this space is the balance between molecules released into and removed from the extracellular space. Release is due to "leakage" or spillover from synap-

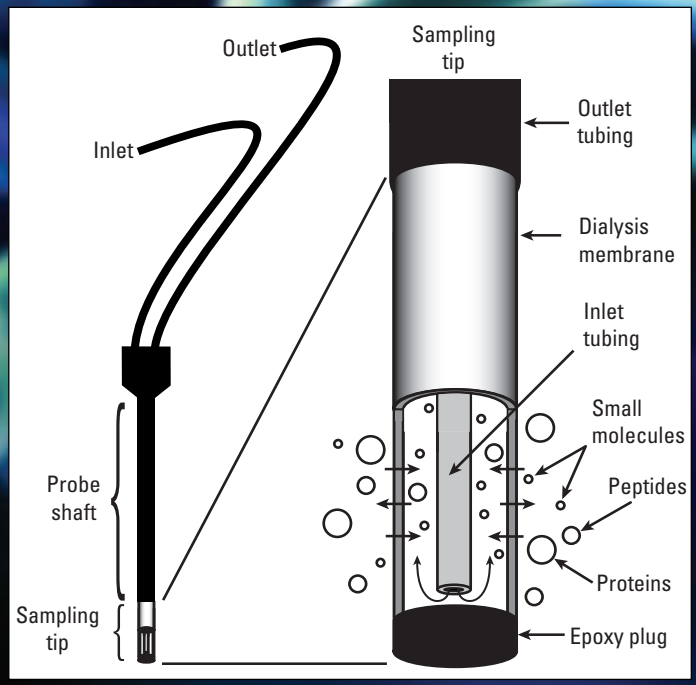
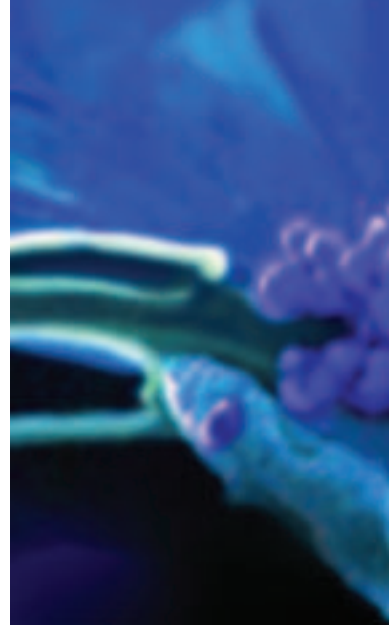


FIGURE 2. A concentric microdialysis probe.

A solution that has an ionic composition similar to that of the extracellular fluid in the brain—yet does not contain analytes of interest—is pumped into the inlet of the probe, flows past the active area of the dialysis membrane, and then flows out the outlet of the probe. As the fluid passes by the dialysis membrane, a concentration gradient is established across the membrane. It facilitates the diffusion of compounds of interest from the extracellular space through the membrane and into the perfusion stream for analysis. Only compounds below the molecular-weight cutoff of the membrane can diffuse through the membrane. Pharmacological challenges can be locally administered by being added to the perfusion solution. This will deliver the challenge to the tissue surrounding the probe by reverse dialysis. Probe tips are typically 200–400 μm in diameter with an active length of 1–4 mm.



ses and direct release of contents into the extracellular space via various mechanisms (Figure 1). Therefore, any conclusions about chemical events within a synapse must be inferred. Presumably, measurements taken in the extracellular space more directly reflect volume transmission than wired transmission.

Interpretation of the results obtained by sampling this environment can be complicated for several reasons. Tissue damage may be associated with the probe's insertion. Active control of neurotransmitter concentrations by release and reuptake within the extracellular space, as well as other environmental factors, may influence the recovery by the probe. And some analytes might come from multiple sources, such as the bloodstream and non-neuronal cells. These factors have resulted in some controversy in interpreting microdialysis data. We will review this controversy with an emphasis on developments since the previous reviews on microdialysis (*10–12*, *S17–S20*) and the validity of this technique (*13*, *14*, *S21*).

Tissue damage. Inserting any structure into the brain results in trauma to surrounding tissue, including both immediate damage, such as edema, and tissue responses that develop over longer periods, such as inflammation and gliosis. Initial studies of the damage caused by inserting microdialysis probes found that blood flow and glucose metabolism decreased near the probe immediately after implant (within 2 h) but recovered to nearly normal levels within 24 h (*15*). Several histological studies of the tissue damage associated with dialysis probes implanted for 1–3 d have found both regions of apparently normal neurons and regions of damaged or degenerating neurons in the immediate vicinity of the dialysis probe (*16–19*, *S22–S29*).

Although the damage is often described as minimal or occurring only within a few cell layers, only one semiquantitative study has been reported (*16*). This study revealed an ~50% decrease in neuronal and synaptic density at the edge of a probe that had been implanted for 40 h. Other signs of damage, such as inflammation and gliosis, were also evident. This report also demonstrated a gradient of damage moving away from the probe: Tissue disruption, including swollen axons, was observed 1.4 mm away from the probe. However, the neuronal and synaptic densities were not different from those of the control tissue at this distance. Another form of tissue damage is impairment of the blood–brain barrier, presumably because blood vessels break as the probe is pushed through the tissue. Most studies have demonstrated that the blood–brain barrier becomes intact again within a few hours of probe insertion (*15*, *20*, *S30*), but this finding is not universal (*21*).

Several days after implantation, glial cells encapsulate the probe; this probably accounts for the inability to record neurochemical responses for more than a few days after probe implantation (*18*). One way to allow measurements over longer periods is to use a guide cannula and insert probes multiple times (*22*). This procedure has been reported to induce moderate glial reaction, which does not completely block the sampling area and even allows neuronal regrowth near the dialysis probe. Stable neurotransmitter measurements were made for 23 d with this approach.

From these studies we can conclude that damage occurs upon implantation of a microdialysis probe and that this damage will depend on the amount of time that has elapsed since the insertion. Other studies suggest that tissue damage may vary with the probe dimensions (*23*), probe material (*18*), sterility of the probe (*17*), and perfusion fluid (*24*). A better understanding of these factors should help to further minimize the impact of the probe and make chronic implants possible.

Microdialysis and functioning neurons. Although tissue damage occurs with insertion or implantation of dialysis probes, several lines of evidence support the conclusion that microdialysis samples the chemical output of functional neurons. The dialysate concentrations of most neurotransmitters are sensitive to tetrodotoxin (TTX); are Ca^{2+}-dependent (information regarding the significance of these observations can be found in the caption for Figure 1); increase with direct neuronal depolarization; and can be manipulated pharmacologically, as expected for functional neurons (*25*, *S17*, *S31–S36*). (As we will discuss later, the amino acid neurotransmitters glutamate and γ-aminobutyric acid [GABA] present a more complicated situation.)

The viability of cells surrounding the microdialysis probe is also demonstrated by studies showing that pharmacological agents perfused through the probe can affect behavior (*26–28*, *S37–S38*). Even more compelling are the studies that demonstrate that the manipulation of the neurotransmitter concentration by local applications of drugs also alters behavior that was previously correlated to the neurotransmitter (*6*, *29*, *S39–S41*); the adenosine sleep study is one example (*4*). Finally, in studies in which electrophysiological electrodes were implanted near microdialysis probes, neurons that appear to have normal electrical properties have been detected (*28*, *30*). All of these results are consistent with the histological studies that show intact neurons in the vicinity of the probe. They also support the conclusion that dialysis probes can sample the neurochemical activity of neurons and that this activity can be related to physiology and behavior.

Timing of experiments. The observations that tissue damage and subsequent responses change with time suggest that the time elapsed after implantation may affect the experimental results. Most investigators have observed that after the probe is inserted, dialysate levels for neurotransmitters require 1–2 h to stabilize. Despite this tendency to stabilize, some changes in responsiveness can be observed. For example, dopamine concentrations decrease only partially in response to TTX infusion for several hours after implantation, but after ~24 h they may be decreased to nearly zero by TTX (*31*, *S42*, *S43*); this finding suggests that there is normalization of neuronal release by then (Figure 1 caption). Because of this result, and the full recovery of blood flow after 24 h, many researchers advocate inserting the probe, wait-

ing overnight, and then conducting the experiment. However, other results suggest that this may not always be necessary because tissue can recover somewhat within a few hours (*32*, *33*).

Comparisons with other methods

Although tissue damage occurs upon insertion, the ultimate consideration is how such damage affects the experimental results. Unequivocal behavioral and pharmacological studies provide evidence that sampling from functioning neuronal circuits is possible. Another way to determine whether artifacts from tissue damage or other sources affect microdialysis is to compare the results and conclusions from microdialysis experiments with those from other types of experiments that presumably suffer from fewer, or at least different, artifacts. For example, it has been suggested that the smaller size of microelectrodes means that they perturb the tissue less than a microdialysis probe and that the microelectrode results are thus less affected by tissue damage (*34*–*36*). Because dopamine can be readily measured by PET, microelectrodes, and microdialysis, it provides the richest ground for comparison.

Basal concentrations of dopamine measured using microdialysis with no-net-flux calibration are in the range 2.5–15 nM (*19*, *22*, *37*, *38*, *S44*, *S45*; these values have been revised to 30–90 nM in some models). In comparison, pulse voltammetric methods have recorded dopamine concentrations of 1.5–26 nM (*39*, *40*). (Anesthetized rats were used in these studies, and in Reference 40, pargyline [which inhibits monoamine oxidase] was used to prevent interference from dopamine metabolites. These pharmacological manipulations may alter dopamine levels.) Modeling studies that used kinetic parameters derived from voltammetric measurements and known firing rates of dopamine neurons have led to estimates of 6–50 nM for dopamine basal concentrations (*12*, *41*). (These models assume that the release of dopamine is only from firing neurons. If other sources of release are present, then such models would underestimate basal concentration [*42*, *43*].) These findings are therefore of the same order of magnitude as measurements made by microdialysis.

Greater differences have been found when fast-scan cyclic voltammetry (FSCV) is used to determine basal levels. The FSCV technique typically has not been used for obtaining basal concentrations because of uncertainty over contributions to background current and the reliance on background subtraction. These limitations can be circumvented by measuring the decrease in FSCV signals after pharmacological manipulations. These experiments have reported decreases of dopamine concentration of 0.5–2.0 μM over 5–20 min (*42*, *44*, *45*). Because the changes are decreases from basal conditions, these estimates would have to be considered the lower limits of basal concentration. The discrepancy between these and other measurements is still unresolved by researchers. This inconsistency has been attributed to greater tissue damage caused by the larger microdialysis probe or to deficiencies in microdialysis calibration, although this explanation does not account for the difference with other microelectrode measurements (*42*).

Yet to be examined is the possibility that artifacts in FSCV measurements contribute to the discrepancy. Such a possibility seems significant in view of recent studies that have urged caution in interpreting basal concentration fluctuations measured using FSCV because changes in current at the redox potential for dopamine can be caused by other redox-active species and by changes in ionic composition, such as pH (*46*, *47*). Dopamine changes can be extracted using principal components analysis (PCA) when multiple known species contribute to the signal, but even this approach is limited by the possibility that unknown species cause distortion of the voltammograms. These studies also reveal that if >90 s elapse between collection of the signal and background voltammograms, background drift results in significant uncertainties in the measurement. In the studies in which large changes in dopamine concentration were reported, relatively long intervals occurred between background and signal voltammograms; furthermore, the PCA method was not used. It may be useful to consider these possibilities and other potential artifacts when trying to resolve the disparate concentration measurements.

These measurements allow researchers to investigate how the intact network **regulates neurotransmission and how behavior correlates to** brain chemistry.

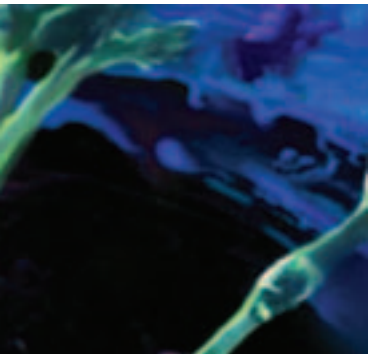
Regulation of in vivo dopamine concentration has also been investigated by multiple methods. Microdialysis measurements routinely show robust increases in dopamine after the administration of dopamine uptake inhibitors, such as cocaine or nomifensine. Both PET (*48*, *49*) and microelectrode (*40*, *47*) studies have also recorded increased basal levels with uptake inhibitors. One possibility is that microelectrodes may detect a quantitatively larger effect due to uptake inhibitors, perhaps because of differences in spatial resolution of the techniques (*47*); however, pulse voltammetric methods result in uptake-inhibitor-evoked changes that are more similar to those of microdialysis.

Another possibility is that microelectrodes allow changes in uptake to be detected sooner after uptake inhibitors are given, generating a slightly better correlation to changes in behavior evoked by the drugs (*50*); however, this conclusion was based on microdialysis measurements taken at 20-min intervals, and higher-temporal-resolution measurements, possible with CE (*51*), are expected to ameliorate such differences. Finally, dopamine agonists are expected to inhibit dopamine release, and this has been observed by both microdialysis and microelectrodes (*52*, *40*).

The above measurements could be considered steady-state measures of dopamine concentration and regulation. Differences in FSCV and microdialysis under dynamic conditions have been observed in an experiment that used simultaneous measurements of dopamine with electrodes and sampling probes spaced at different distances from each other. In this experiment, electrical

stimulation increased dopamine concentrations detected at electrodes far from the dialysis probe but not at electrodes next to the probe (*35*, *36*). In the presence of uptake inhibitors, dopamine could be detected at electrodes, regardless of the distance from the probe. These results led to the conclusion that damage caused by the dialysis probe prevents impulse-dependent release from occurring in the vicinity of the probe. This conclusion is at odds with microdialysis studies that have recorded TTX-sensitive basal levels (*S31*) and increases in dopamine efflux during electrical stimulation (*53*, *S46*–*S48*). Differences in the experimental conditions may have led to this discrepancy. One potentially significant difference is that the electrode measurements were made soon after insertion, but a recovery period is required to achieve TTX-sensitive results for dopamine. It would be interesting to determine the extent of impaired release near a dialysis probe with longer implant times.

Comparative measurements of other neurotransmitters are not as readily available. Basal concentration of glutamate in the brain has been estimated to be 1.8–5.0 μM by using microdialysis with two different in vivo calibration methods (*54*, *S49*, *S50*). These concentrations are similar to those observed by directly sampling the extracellular space and using low-flow push–pull perfusion, both of which use probes smaller than a microdialysis probe (*55*, *56*).



Enzyme-modified amperometric sensors for glutamate provide another opportunity for comparison (*57*, *S51*, *S52*). Difficulties with calibration and uncertainty over nonspecific contributions to background signals have prevented the determination of basal concentrations strictly on the basis of the currents measured upon implantation. However, as with the dopamine measurements, microinjection of pharmacological agents that may suppress glutamate release have been used to approximate the lower limit of basal concentration. Two studies have reported that the microinjection of TTX lowers the signal by amounts corresponding to 1–3 μM and 30 μM, respectively (*58*, *59*). Although the former measurement would result in an estimate of the glutamate basal concentration that is comparable to that obtained by sampling techniques, the latter concentration would be substantially higher. However, the difference between electrochemical and sampling results is not as great as that observed for dopamine. Regulation by uptake inhibitors seems to be qualitatively similar when measured by both methods.

Microdialysis, PET, and microelectrode measurements agree in most ways with regard to trends and regulation of dopamine and glutamate; however, some quantitative differences have been found. Although the greater tissue damage caused by larger probes may account for some of these differences, many other factors—such as spatial resolution, temporal resolution, anesthesia, and time since implantation—must also be considered. Tissue damage caused by microdialysis probes should be kept in perspective. Although microelectrodes cause less damage, they also measure from a smaller area and may experience some artifacts as a result, even though no evidence of this has been found. Furthermore, it can be argued that synaptosomes, brain slices, and cultured cells all create a significantly more artificial or "damaged" environment than implanted probes, yet significant advances in our understanding of neurotransmitter release and regulation have been made by use of these preparations. The many successes of microdialysis for in vivo investigations, as well as the general agreement with other methods, suggest that tissue damage does not preclude valid measurements.

Recovery and probe calibration

The methods of calibration and in vivo quantification are also sources of controversy in the interpretation of microdialysis data. When a probe is placed in a sample and perfused, the concentration in the dialysate (C_{out}) will be less than that in the sample (C_{ext}). The ratio C_{out}/C_{ext} is the relative recovery (R) and increases as the flow rate decreases, the probe's active area increases, or the temperature increases. The relative recovery will also be influenced by probe geometry, adsorption of analytes to the membrane, and the molecular-weight cutoff of the membrane. These factors can be accounted for by in vitro calibration wherein known concentrations of analyte are sampled and C_{out} is measured, allowing calculation of R (*20*).

It has long been recognized that R in vitro cannot be used for in vivo calibration with accuracy because of the effect of the brain environment on R (*60*, *61*). In vitro, the probe is exposed to a large volume of a homogenous solution with unimpeded diffusion of analyte to the probe. In contrast, in the brain, only the small extracellular fraction is sampled, and molecules must diffuse through the tortuous extracellular environment to reach the probe. Furthermore, molecules diffusing toward the probe may be transported into cells or metabolized.

Given the problems with in vitro R, several in vivo calibration methods have been developed, including no net flux (NNF; *22*, *S53*–*S55*), variable flow rate (also called extrapolation to zero flow rate; *S54*, *S56*), and low flow rate (*62*, *63*, *S56*, *S57*). The most popular method has been NNF, in which the analyte of interest is perfused at various concentrations through the probe, and the concentration of this analyte is then measured at the outlet of the probe. Perfusion concentrations are chosen to bracket the actual concentration of the analyte in vivo (C_{ext}). According to the theory of Bungay et al. (*64*),

$$C_{in} - C_{out} = E(C_{in} - C_{ext}) \quad (1)$$

in which C_{in} is the concentration added to the perfusion fluid, and E is the "extraction efficiency", which is a measure of the ability of the tissue to take up material from the probe. Thus, a plot of $C_{in} - C_{out}$ versus C_{in} yields a straight line (Figure 3a). The point of NNF across the membrane is the x intercept where $C_{in} = C_{out}$. The desired C_{ext} can be determined from this point because Equation 1 reduces to $C_{in} = C_{ext}$ when $C_{in} - C_{out} = 0$. A potential contribution to error with the NNF method is nonlinear tissue effects; however, a recent theoretical analysis has demonstrated that C_{ext} determined by NNF is insensitive to non-

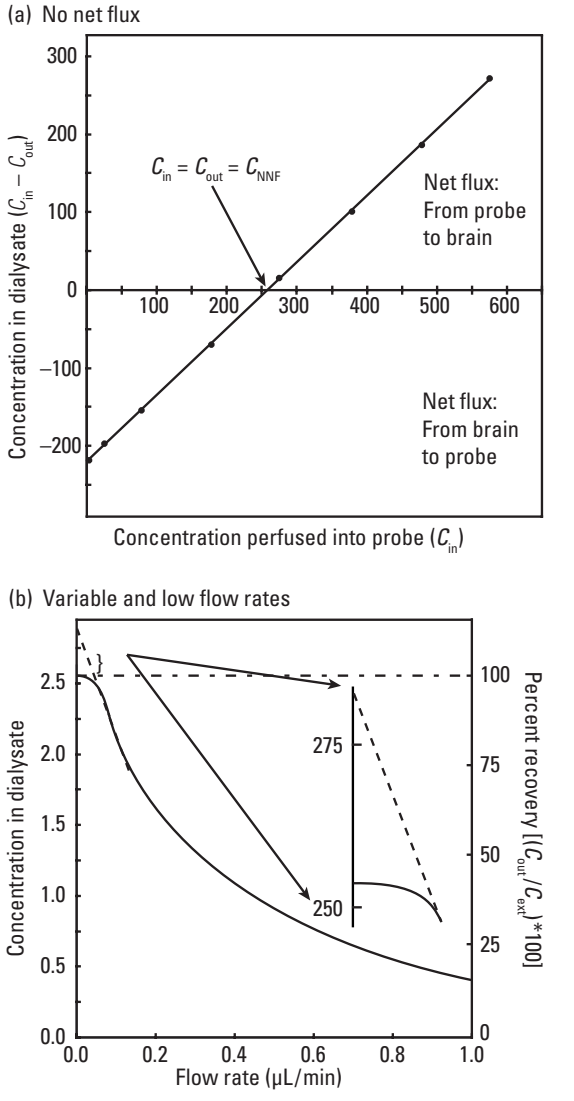


FIGURE 3. Quantitative microdialysis.

(a) In the NNF calibration method, the analyte is added to the perfusion fluid at different concentrations (C_{in}), and the concentration of analyte in the dialysate (C_{out}) is measured. Data are plotted as $C_{in} - C_{out}$ vs C_{in}. The point at which $C_{in} = C_{out}$ is the point of NNF and represents the extracellular concentration (C_{ext}) of the analyte. The slope of the curve is usually considered to equal *E*. (b) In the variable-flow-rate technique, the flow rate is varied, and the concentration in dialysate is measured. Relative recovery increases as the flow rate decreases, reaching a point at which $R = 100\%$. This approach may overestimate the extracellular concentration when the curve is extrapolated to zero flow rate (inset). Low-flow-rate methods typically use flow rates <100 nL/min, which give recoveries at or near 100%, such that $C_{out} = C_{ext}$.

linearities in uptake (due to Michaelis–Menton kinetics) or release (due to autoreceptor effects) (*65*).

Another potential complication with the NNF method is that it assumes that *R*, the ability of the probe to collect from the extracellular space, and *E*, the ability of the probe to deliver to the extracellular space, are the same. (This can be seen when $C_{in} = 0$. In this case, Equation 1 reduces to $C_{out}/C_{ext} = E$, thereby making *E* equivalent to *R*.) A model that allows for a difference between *E* and *R* results in the following equation to describe difference plots: $C_{in} - C_{out} = E[C_{in} - (R/E) \times C_{ext}]$. This relationship implies that one cannot estimate C_{ext} without knowing both *E* and *R* (*35*, *66*, *67*). Consideration of *E* and *R* separately has led to studies examining whether they actually differ.

The simultaneous FSCV and microdialysis studies mentioned earlier provide some evidence for such differences (*35*, *36*). Specifically, when uptake was inhibited, electrically stimulated overflow was enhanced more near a dialysis probe than away from it; thus, *R* appears to have increased at the probe with uptake inhibition. Because *E* measured by microdialysis had previously been shown to decrease with similar treatments in other studies, this finding implied that *E* and *R* differed and could actually move in opposite directions with pharmacological treatments. This possibility would confound the interpretation of NNF calibrations. Analysis of other comparisons of FSCV and microdialysis data, however, has reached the opposite conclusion: that *R* decreased with uptake inhibition, similar to *E* (*12*). Studies that measure the values of *E* and *R* in the same experiment may be required to better determine whether this condition actually occurs.

A physical mechanism of how *E* and *R* may come to differ is not known, though efforts have been made to uncover one. Two theoretical studies have concluded that the presence of discrete sites of release does not contribute to a difference in *E* and *R* and, therefore, does not have a significant effect on the concentration estimated by NNF (*68*, *69*). It has been suggested that if tissue damage caused by probe implant selectively impaired release relative to uptake within a layer around the probe, then *E* and *R* would be different (*69*). In such a case, dopamine released outside the damaged region would be unlikely to diffuse through the uptake-active region and be collected by the probe; this would result in low concentrations of dopamine in dialysate. This consideration led to revision of models of mass transport to the probe (*68*, *69*). Both models show that selective impairment of release relative to uptake around a probe will cause the NNF method to underestimate basal concentrations.

When dopamine was used as a model system and semiquantitative assessments of loss of release sites were incorporated, the models predicted that NNF would underestimate concentrations by as much as 90%; however, such estimates are highly dependent upon accurate knowledge of the relative impairment of release and uptake. If tissue damage is accompanied by degradation of both release and uptake sites—that is, formation of a passive layer with reduced release and uptake—the NNF method is not affected (*68*, *69*). Therefore, confirmation of the relative impairment of release and uptake will be necessary to determine the effect on NNF calibrations.

In vivo concentrations could also be estimated by using the low-flow-rate method or the variable-flow-rate method (Figure 3b). The underlying assumption of these methods is that if a probe had no flow through it, its internal concentration would eventually equilibrate with the extracellular concentration. At low

Mitigating tissue damage and **developing a better understanding** of the effect of **inevitable tissue damage** on the measurement **would be valuable.**

flow rates, typically <100 nL/min, recovery approaches 100%, giving rise to a quantitative estimate of extracellular concentration (*S50*, *S54*, *S56*, *S57*). One reason that this method is less popular is that conventional analytical techniques such as HPLC require long times to collect sufficient sample at such low flow rates; however, sensitive methods like CE alleviate this situation. In addition, longer probes, which are feasible with larger subjects, such as humans, increase recovery and allow the same effect to be achieved with higher flow rates and more convenient sample sizes (*70*). The effects of issues raised for NNF on these calibration methods are not clear.

Although absolute quantification is sometimes a goal, most studies rely on quantifying relative changes, rather than absolute changes; this obviates the need for in vivo calibration. In such measurements, it is understood that the concentration in dialysate reflects the balance of release and uptake such that a change in either parameter might alter measured levels.

Glutamate and GABA in dialysate

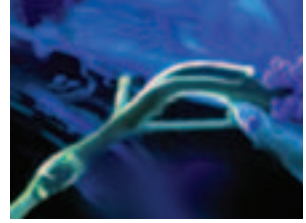
The basal concentrations of most neurotransmitters measured by microdialysis are TTX-sensitive and Ca^{2+}-dependent, as expected based on known properties of neurons. However, basal concentrations of glutamate and GABA are not reliably regulated by TTX or Ca^{2+}. (In the case of GABA, TTX sensitivity has recently been reported with new HPLC methods, but the TTX effect was slow in developing, unlike the rapid decreases seen with other transmitters [*S65*].) These observations have resulted in controversy over the meaning of measurements of these compounds.

A commonly held view is that neuronally derived glutamate and GABA do not escape the synapse because of strong uptake mechanisms that prevent their mass transport from the synapse (*S66*). This view is supported by electrophysiological studies that imply that glutamate has a minimal ability to diffuse from synapse to synapse under low firing conditions; it is also supported by anatomical studies that show that glutamate synapses tend to be enveloped by astrocytes bearing glutamate transporters (Figure 1b; *25*, *S58*, *S67*). If glutamate and GABA basal concentrations are not due to spillover from synapses, then what is the source of the micromolar concentrations observed in dialysis samples? It has recently been found that a substantial fraction of the dialysate glutamate levels are maintained by the cystine–glutamate antiporter, a protein preferentially located on astrocyte membranes (*S68*). Astrocytes may have other release mechanisms as well. It has also been suggested that astrocytes surrounding synaptic junctions experience increases in intracellular Ca^{2+} concentration when glutamate is transported into the astrocyte or binds to glutamate receptors located on the astrocyte and near the synapse. This increase in Ca^{2+} levels induces a release of glutamate into the extracellular space, possibly as a method of volume transmission (*25*, *S69*). The astrocyte may, therefore, form a tripartite synapse with the pre- and postsynaptic neurons and work to modulate the synapse or even enable lateral signaling between synapses (*S70*, *S71*). Similar astrocytic regulation of GABA levels was suggested in these and other reports (*S65*). These new insights suggest that astrocytes play a greater role in synaptic activity than previously thought and offer an intriguing rationale for the difficulty of detecting neuronally derived glutamate and GABA by microdialysis (*S68*, *S72*).

Although basal levels of glutamate and GABA measured by microdialysis are apparently not governed by direct spillover, ample evidence exists that increases in neuronal activity lead to increased dialysate concentration of these amino acids. Electrical, pharmacological, and behavioral activation of glutamatergic tracts has revealed TTX-sensitive and/or Ca^{2+}-dependent increases in glutamate in the terminal area (*32*, *S55*, *S59*, *S60*). One interpretation of such results is that glutamate release is sufficiently large to overwhelm uptake under these conditions and allow detection of spillover. Such a result would be consistent with electrophysiological studies that have demonstrated spillover of glutamate under high use conditions (*S73*). The release observed may also be from neurons mediated by astrocytes through mechanisms discussed above; however, this has yet to be resolved.

As mentioned previously, glutamate microsensors have shown TTX sensitivity in basal levels of glutamate. The discrepancy between this result and the lack of TTX sensitivity for microdialysis may reflect the fact that the higher spatial resolution of electrodes allows them to get closer to glutamatergic synapses. In addition, differences in the delivery of TTX for these types of experiments may affect the results. For microelectrode studies, high concentrations of TTX are microinjected next to probes, whereas in microdialysis, lower concentrations are delivered by reverse dialysis. Nonspecific effects of TTX at high concentrations could lead to differences in the results.

Regardless of the source of glutamate and GABA measured in microdialysis samples, another question is whether the molecules detected outside the synapse have any functional consequence. Because glutamate and GABA receptors have been detected well removed from the synapse, it is reasonable to expect that measurements made in extrasynaptic space would reflect the concentration seen by those receptors. Indeed, ample evidence supports the hypothesis that glutamate and GABA in the extracellular space, as detected using microdialysis, activate these receptors, with functional consequences. Concentrations of the transmitters in the extracellular space can be correlated with behavior, and pharmacological manipulations that alter receptor function also alter behavior (*6*, *S74*). Thus, although the cellular source of amino acid neurotransmitters measured using microdialysis cannot always be definitely determined, the concentrations measured seem to reflect neuronal activity, regulate neurons, and affect behavior.



Better probes

Microdialysis sampling provides significant insights into brain function when appropriate care is taken in interpreting results. As a result, in many laboratories, microdialysis will remain an important workhorse for understanding the brain. Nevertheless, improvements are welcome. Investigation and resolution of controversies surrounding the use of microdialysis will lead to a greater knowledge of this method and possibly expand its application. Mitigating tissue damage and developing a better understanding of the effect of inevitable tissue damage on the measurement would be valuable. Development of miniaturized sampling probes, such as low-flow push–pull perfusion, and direct sampling offer promising alternatives to conventional microdialysis sampling (*55*, *56*). These probes have sampling tips that are similar in size to microelectrodes; therefore, they improve spatial resolution by as much as 500-fold and may cause less tissue damage. At the same time, they maintain the significant advantages of microdialysis sampling, such as versatile, multicomponent chemical monitoring. Because the tiny samples generated by these probes require high-sensitivity techniques and improved methods of sample handling, further development is required.

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