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Electrochemical monitoring of biogenic amine neurotransmission in real time

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Abstract

Three techniques, constant-potential amperometry, high-speed chronoamperometry, and fast-scan cyclic voltammetry, have been used extensively to investigate the rapid events associated with neurotransmission. These techniques vary in sensitivity, chemical resolution and temporal resolution. Amperometry provides the best temporal resolution but little chemical resolution. Fast-scan cyclic voltammetry provides both good temporal and chemical resolution, while high-speed chronoamperometry offers good temporal resolution and moderate chemical resolution. The amount of chemical information which is needed for a neurochemical measurement depends upon the sample. For single cells, secondary methods, such as HPLC and capillary electrophoresis, offer extensive chemical information about the contents of a cell. With this information, chemical information is not needed during the electrochemical measurement. Therefore, amperometry is employed to obtain the greatest temporal resolution. However, when using more complex biological samples, such as brain slices or in vivo implantation, there is a greater demand for chemical resolution provided by the electrochemical measurement. To bolster results, further confirmation is sought from anatomical, physiological and pharmaceutical evidence. Within this review, the three electrochemical techniques are outlined and compared. Examples are then provided of measurements which have been made in the three predominant biological samples which have been studied: single cells, brain slices and intact animals.

Keywords: Fast-scan cyclic voltammetry; Carbon fiber microelectrodes; Neurotransmitters; Biogenic amines; Amperometry; High speed chronoamperometry

1. Introduction

For several years, a variety of electrochemical techniques have been developed to monitor neurotransmitters as they perform the job of intercellular communication [1,2]. These techniques differ considerably with respect to their sensitivity, chemical selectivity and temporal resolution. In recent years, three techniques have become predominant and this review will focus on them. They are constant-potential amperometry, fast-scan cyclic voltammetry (FSCV) and high-speed chronoamperometry. In this review we summarize

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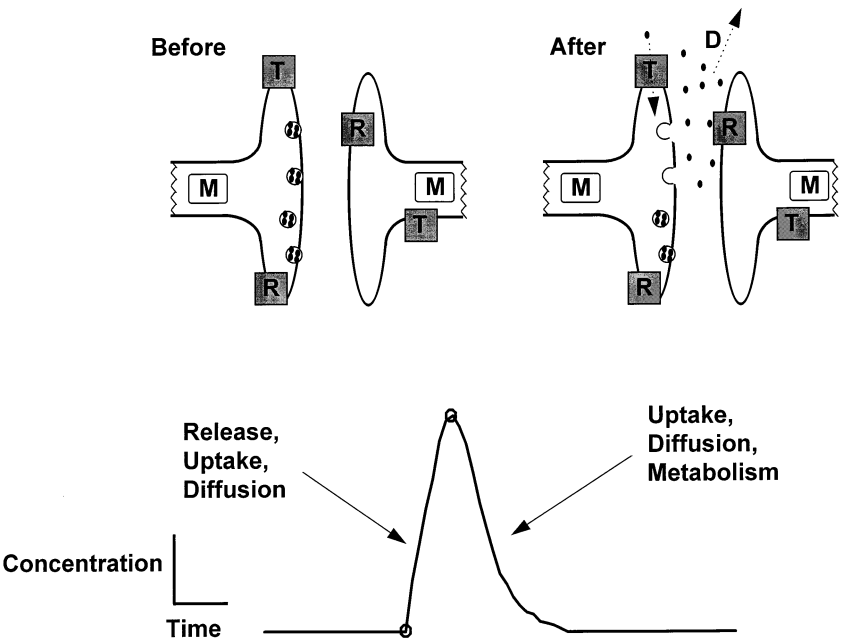

Fig. 1. Local events during neurotransmission. (Upper panel) The cartoon on left illustrates a synapse prior to release. A number of neurotransmitter vesicles are near the cell membrane awaiting release. The boxes labeled with 'T' represent transporters and those labeled with 'R' represent receptors. The internal boxes labeled with 'M' represent intracellular metabolism. At right, a simple illustration shows that some of the vesicles have released their contents following depolarization. Once released, the neurotransmitter can diffuse away from the synapse, interact with receptors and/or be uptaken by the transporter. (Lower panel) The curve represents the neurotransmitter concentration changes that occur at the surface of a nearby working electrode. The superimposed boxes represent the start and finish of the stimulation. During the stimulus, three processes influence the concentration: release, uptake and diffusion. Following the stimulation, uptake and diffusion continue to be important while release is assumed to cease. On the short time scale of most electrochemical experiments metabolism does not influence the extracellular concentration of neurotransmitter.

the characteristics of each of these techniques, indicating their strengths and weaknesses. In addition, examples are presented of the new insights which measurements with these techniques have provided to synaptic transmission.

1.1. Synaptic transmission

Neurons are specialized cells that collect, integrate, and relay information between different parts of the body. Information within neurons is conveyed by electrical means—transmembrane potentials that have classically been studied by the techniques of electrophysiology. Communication between neurons relies primarily on secretion of small molecules from a neuron as a result of the intraneuronal electrical activity, and subsequent interaction of the neurotransmitters with receptors located on neuronal membranes. The response of a receptor to a neurotransmitter depends on the concentration of neurotransmitter that is adjacent to it and the duration that it is present. Because electrodes for voltammetry can be made quite small, they can be placed in the extracellular fluid and used to measure these events with little damage to the surrounding tissue. Only molecules that are easily oxidized or reduced can be detected with this approach, but this includes a list of several molecules with recognized physiological importance. This includes the catecholamines (dopamine (DA), norepinephrine (NE), epinephrine (E)) as well as 5-hydroxytryptamine (5-HT), and histamine (His), a group collectively termed the biogenic amines in this review.

Neurotransmitters are typically stored within vesicles which are close to the cell membrane (Fig. 1). Once released from the cell, they can interact with transporters as well as receptors. The transporters, also membrane-bound proteins, can transport extracellular neurotransmitter back into the cell, a process termed uptake, and terminate the chemical signal. The ability to make real-time measurements of neurotransmitters allows several questions to be answered concerning the microscopic steps of this release and uptake. For example, the presence of vesicles at the terminal suggests that release is a quantized event. We can then ask, how large are those units, what controls the kinetics of vesicular opening, and is there only a single type of neurotransmitter present in each vesicle? Once neurotransmitter is released from the cell, still other questions can be asked. What is the half-life of a neurotransmitter after release? Is the half-life for a neurotransmitter the same throughout a complex organ like the brain? Is it possible for neurotransmitter to exit the synapse following release? Are extrasynaptic concentrations important? What is the effect of pharmacological agents on these processes?

To understand how these questions can be approached with electrochemistry, it is important to understand these techniques.

2. Electrolytic techniques

2.1. Introduction

Electrolytic techniques are a subdivision of electrochemical techniques that involve the oxidation or reduction of analyte molecules at the surface of an electrode as a result of an applied potential. Whether a molecule is a candidate for these approaches can be determined from its formal reduction potential. The formal potentials of several small molecules important in the brain have been tabulated [3]. The formal potential must fall within the 'electrolytic' window of physiological solutions. When the applied potential is beyond these bounds, the solvent is oxidized or reduced. Even if the formal potential of an oxidation or reduction falls within the electrolytic window, it still may not be possible to use electrolytic techniques to monitor the molecule. The rate of electron transfer between the electrode and molecule is a potential dependent process, and limits how fast an oxidation or reduction can occur [4]. If this rate is slow, poorly behaved electrochemistry will result, and the electrochemical response will occur at a potential other than the formal potential. Electron transfer rates are a function of both the molecule and the electrode material.

The electron transfer rates for the biogenic amines at carbon electrodes are moderately fast. This means that the potential of their electrochemical responses, although not at the formal potential, are not shifted a great deal. Indeed, because of the combined effects of the formal potential and the kinetic processes, the potential of these responses can provide considerable information concerning the identity of the species detected.

Electrolytic oxidation of a solution phase molecule occurs at the electrode surface. Thus, electrolytic techniques are also affected by the way in which material reaches the surface, i.e. the mode of mass transport. For the experiments described in this review mass transport is controlled by diffusion, the random motion of molecules in solution. The rate of consumption by electrolysis at the electrode surface is termed flux, and it determines the magnitude of the current. Consumption of a molecule at the electrode surface generates a concentration gradient, $(dC/dx)_{x=0}$, and the flux is proportional to this gradient as stated by Fick's first law. The proportionality constant is the diffusion coefficient for the molecule and the length of the concentration gradient is often referred to as the diffusion layer. Thus, the magnitude of the current arising from the oxidation of a solution phase molecule, termed the faradaic current, is determined by the electrode potential and the rate of diffusion of molecules to the surface.

In addition to the faradaic current, other currents also arise in electrochemical experiments, and these must be distinguished to resolve the contribution from the molecule(s) of interest. These additional currents are associated with processes at the surface of the electrode. Typically,

the largest component is the charging current that arises whenever the potential of an electrode differs from that of solution. The solution 'reacts' by creating a double layer of charge. Within the double layer, dissolved ions will arrange to balance the excess charge present on the electrode surface. The charging current is proportional to the capacitance of the electrode, which is, in turn, proportional to the area of the electrode. A second component of surface-associated current involves reduction and oxidation of surface attached species. These may be covalently attached functional groups or adsorbed molecules. For example, a carbon fiber microelectrode typically has several types of electroactive oxides on its surface [5]. Additionally, a number of cations, including dopamine, will adsorb to the surface of the electrode.

An important feature of surface-associated currents is their transient nature. The charging current at an electrode decays rapidly, especially at microelectrodes. The time constant for this decay is the product of the uncompensated solution resistance and the electrode capacitance. Additionally, because of the proximity of covalently attached and adsorbed species to the electrode surface, the oxidation or reduction of these groups is limited only by the kinetics of the reaction. This behavior is in contrast to the faradaic current which is a function of mass transport in solution.

The response of both the faradaic and surface-associated currents depends on the potential waveform applied to the electrode surface. This feature distinguishes the techniques described in the following section and leads to their individual strengths and weaknesses.

2.2. Constant-potential amperometry

The simplest electrolytic experiment is constant-potential amperometry (Fig. 2A). In this experiment the potential is selected so that the surface concentration of the analyte of interest will be driven to zero by the electrolysis process. This ensures that consumption by the electrode is rapid producing a current which is limited by the mass transport rate. Because the electode potential remains fixed, the surface-associated currents are virtually eliminated following the initial decay time. This is an important consideration for digital acquisition of data. When there is no surface current, the complete dynamic range of the system can be used to acquire high-resolution signals due to analyte oxidation or reduction.

Because the current is directly proportional to concentration at all times, amperometric currents may be measured continuously at the electrode. Thus, amperometry offers the best temporal resolution of any electrochemical technique and is the most desirable technique to use to follow the rapid chemical changes that occur during many important neurochemical events. Ultimately, it is filtering, signal-to-noise ratio and sampling rate which will determine the temporal resolution of amperometry rather than the response time of the probe.

Unfortunately, amperometry has very little chemical selectivity. If an oxidation or reduction can occur at the final potential of the electrode, it

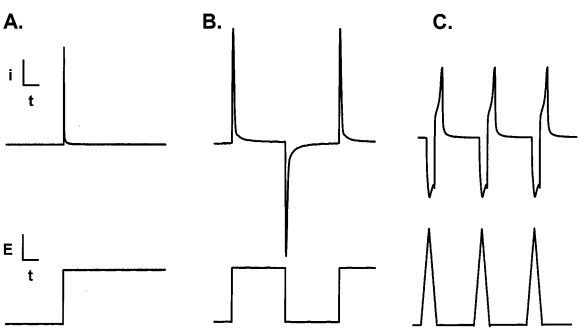

Fig. 2. Voltage waveforms and current responses for voltammetric techniques. (A) Constant-potential amperometry. For constant-potential amperometry, a single voltage step (lower panel) is applied to the electrode producing a transient current which decays rapidly (upper panel). (B) High-speed chronoamperometry. A potential step is applied repetitively to the working electrode with a frequency between 5 and 25 Hz (lower panel). Each potential step produces a transient current (upper panel) similar to that seen with constant potential amperometry. The current does not return to baseline between the potential steps. (C) Fast-scan cyclic voltammetry. A triangle wave (lower panel) is applied repetitively to the electrode, generating a background current (upper panel). There is a delay between each triangle wave, typically nine to ten times the duration of the triangle wave. This time was made much smaller for illustrative purposes.

will. It is impossible to distinguish the component currents when multiple electroactive molecules are present in the same solution. To improve the selectivity of amperometry, surface modifications have been developed. These modifications include incorporation of enzymes [6,7], ion-exchange membranes [8], and inorganic catalysts [9].

A common ion-exchange membrane is Nafion, a polyanionic film which can be coated on the surface of the electrode. Anions such as ascorbate and several metabolic products of neurotransmitters are selected against, and cannot reach the modified electrode surface as easily as a bare electrode surface. A disadvantage of Nafion coatings is the slowed temporal response of the electrode. The degree of temporal distortion is dependent upon the thickness of the Nafion film [10].

Several enzyme modifications have been described. Although enhancement is produced for the substrate of the enzyme, selectivity is not guaranteed since any electroactive molecule which can reach the surface of the electrode can contribute to the measured current. This often makes calibration of the electrode difficult, especially when used in complex environments, such as the intact brain. Inorganic catalysts have been incorporated into electrodes to enhance oxidations and reductions which are not typically fast enough to produce measurable currents. To simplify interpretation of results, these electrodes have been used in simple systems.

When calibrating electrodes used in amperometry it is important that the diffusion layer remains the same as it was in the measurement situation. This means that convection must be minimized in both cases (Fig. 3A), perhaps by the use of a vibration-free table. Measurements in tissue or with cells are usually made with the electrode placed against the secreting surface. This sets the spacing of the concentration gradient, and is difficult to reproduce in a nonbiological environment. For this reason, concentrations are rarely reported from these amperometric experiments.

The presence of high concentrations of other electroactive molecules can also perturb the diffusion layer [11]. For example, ascorbate occurs in the brain between 0.2 and 0.5 mM. Neurotransmitters, such as DA, NE and 5-HT occur at concentrations about 2–3 orders of magnitude less than ascorbate. Because ascorbate is more easily oxidized than the neurotransmitters, the oxidized products of the neurotransmitters will react with ascorbate in solution to regenerate the original neurotransmitter. Thus, the diffusion layer shrinks relative to what it would be without the second electroactive analyte present. Given its influence on current, a similar concentration of ascorbate should be included in the calibration buffer. Similar precautions should be made for any other high-concentration electroactive molecule which is more easily oxidized or reduced than the molecule of interest. If there is not a large concentration difference between the analyte of interest and the more easily oxidized species, then current enhancement is usually minimal.

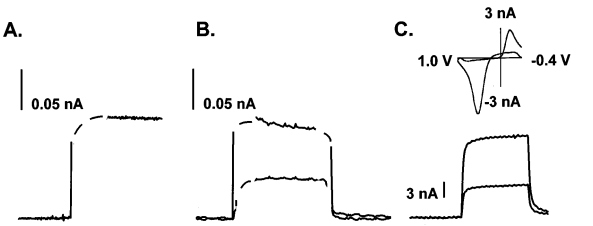

Fig. 3. Flow injection analysis. (A) Responses to catecholamines. Amperometric response of a microdisk electrode to 5 μM epinephrine. The dashed line represents extrapolation of baseline currents to stop flow currents. Before flow is stopped, the current is larger. The electrode potential was held at +650 mV vs SSCE. (B) High-speed chronoamperometric response of a cylinder microelectrode to 2.5 μM dopamine. The dashed line represents extrapolation of baseline currents to stop flow currents. Before the flow was stopped, the oxidative current was larger and the reductive current smaller. The top trace is the oxidative current (550 mV vs SSCE) and the bottom trace is the reductive current (0 mV vs SSCE). (C) Fast-scan cyclic voltammetry response of a cylinder microelectrode to 2.5 μM dopamine. The current represents the average oxidative current between 550 and 650 mV vs SSCE. Above the current trace is the background subtracted cyclic voltammogram.

2.3. High-speed chronoamperometry

High-speed chronoamperometry provides fast electrochemical monitoring with modest chemical resolution. During high-speed chronoamperome-

try, a square-wave potential is applied to the electrode (Fig. 2B). The frequency of the square wave is typically between 5 and 25 Hz. The potentials chosen are typically sufficient to oxidize the analyte of interest and then re-reduce the oxidative product to the original molecule. This re-reduction of the oxidized neurotransmitter is an important advantage over constant potential amperometry since it prevents the accumulation of oxidized neurotransmitter, often a potent neurotoxin. With each potential step, there will be a large surface-associated current. However, given the small time constants of microelectrodes, much of this current will decay rapidly. Thus, it is the current during the last 80% of each step which is measured, providing a large dynamic range for the faradaic portion of the current measurement. All of the current during the last 80% of each step is averaged and recorded. The average current from each step is then plotted as a function of time (Fig. 3B). To provide some degree of chemical resolution, the ratio of the reductive current value, at the time of the oxidative current peak, to the oxidative peak current, is calculated. This ratio is different for several important neurotransmitters. It is important to realize that this ratio is only calculated for one point in time and not throughout the entire trace.

High-speed chronoamperometry probes are typically coated with Nafion to improve the selectivity of the measurement. Calibration of the electrode is possible and commercial systems are readily available. Unfortunately, because some surface-associated current is contained within high-speed chronoamperometry measurements, changes in ionic composition of the solution surrounding the electrode will generate spurious signals.

2.4. Fast-scan cyclic voltammetry

Fast-scan cyclic voltammetry is an unconventional electrolytic technique designed to provide good temporal and chemical resolution. To produce these characteristics, a triangle waveform is intermittently applied to the working electrode (Fig. 2C). The scan rate ($V\ s^{-1}$) and potential limits determine the time required for each triangle wave. Typically, the time between scans is about ten times as long as the time of the scan and determines the temporal resolution of FSCV data. The delay is necessary to prevent successive scans from influencing one another, i.e. to allow the current to remain proportional to the solution concentration of the analyte [12].

Traditionally, current measurements during electrolytic experiments avoid the large surface-associated currents which arise when the potential of the electrode is changed. When sufficient time is allowed following a potential step, the faradaic current can be recorded with little surface-associated current and the highest resolution. However, when the potential is continually varied, the surface-associated current will remain throughout the measurement. This current is often referred to as the background current for the electrode (Fig. 2C).

During FSCV, the largest surface-associated current is due to charging and discharging of the double layer capacitance. The charging current is directly proportional to both the scan rate and the capacitance of the electrode. If the electrode behaved only as a capacitor, the background current would be a square wave, with a peak height equal to $v \cdot C$ [4], where v is scan rate and C is the capacitance of the electrode. However, the electrode is not simply a capacitor. The presence of electroactive functional groups on the surface of the electrode leads to another source of current. Because this current is produced by oxidation and reduction of the functional groups, it is dependent upon the potential of the electrode and does not add uniformly to the background current. Thus, the background current at a typical electrode will have several peaks and valleys the position and occurrence of which depend upon the relevant surface groups and the scan limits of the waveform. These are shown in Fig. 2C. Fortunately, carbon fiber microelectrodes have extremely stable backgrounds. Because the background current is stable, digital subtraction allows any sources of current in addition to the background current to be revealed.

At fast scan rates, the peak current for an analyte current is given by $i = kn^{3/2}AD^{1/2}v^{1/2}C$ [4], where k is 2.7×10^5, n is the number of electrons

per molecule for the oxidation or reduction, A is the area of the electrode, D is the diffusion coefficient, v is the scan rate and C is the bulk concentration of the analyte. This equation determines the practical limit for scan rates at microelectrodes. The analyte current increases with the square of scan rate, but the background current increases with scan rate. If background currents become too large, there will not be sufficient digital resolution to detect the analyte current.

To its advantage, FSCV provides a moderate amount of chemical information. To reveal this chemical specificity, the total current from one point in time is subtracted from the total current at another point in time. The differential current is then plotted against voltage. The result, termed a background-subtracted cyclic voltammogram, acts as a chemical identifier for the analyte (Fig. 3C). It is presently possible to identify concentration changes for several neurotransmitters and ions in solution using background-subtracted cyclic voltammograms, including serotonin, norepinephrine, epinephrine, dopamine, histamine, O_2, H^+ and Ca^{2+}. Unfortunately, it is not possible to readily distinguish dopamine and norepinephrine at fast scan rates.

Because of the small diffusion layer during FSCV, the current measured is perturbed minimally by convection. Therefore, calibration of the electrode is quite simple in a flow injection analysis system. The electrode is placed at the outlet of a flow stream, and a bolus of an analyte is injected past the electrode. The resulting current allows the electrode to be characterized for response time, sensitivity and selectivity, i.e. how the cyclic voltammogram for a particular analyte will appear at a particular electrode. To aid analysis, the peak current, either reductive or oxidative, is plotted as a function of time. Using calibration factors determined with the FIA system, it is possible to convert currents measured during experiments into concentrations.

Regrettably, the large amount of background current inherent to FSCV can lead to differential currents from changes in ion concentrations. Two especially problematic ions for carbon fiber microelectrodes are Ca^{2+} and H^+ [13,14]. Changes in these ions are often associated with neurochemical activity. Changes in these ions cause the background current at the electrode to 'shift' by perturbing the oxidation and reduction potentials of the surface functional groups on the carbon fiber electrode. Fortunately, the background-subtracted cyclic voltammograms due to changes in the concentration of these ions are fairly distinct and easily distinguished from important neurotransmitters.

3. Investigations of neurotransmission

3.1. Single cell experiments

Voltammetric recordings made at single cells allow the release of neurotransmitter to be studied with considerable temporal and spatial resolution [15]. Chemical specificity is often a secondary concern of the electrochemical measurement, once the cell's composition is determined using very sensitive and selective means, i.e. HPLC and/or capillary electrophoresis. Once the contents of the cell are known, it is relatively straight forward to determine what the possible contributors to any electrochemical signals will be. If, for example, a cell type is shown to contain norepinephrine and no other biological analytes with similar oxidation potentials, then it is safe to use amperometry to monitor release with little fear of unknown contributions to the measured current. Having determined the composition of the cell, the analytical contents of the system are defined, precluding the necessity for chemical specificity at the electrochemical level. Additionally, single cells can be isolated on a microscope slide, which allows visual confirmation that the cell is the only contributor to the measured signal. With piezoelectric positioners, spatial resolution is only limited by the size of the electrode. The media surrounding the cell is also controlled, which allows the study of its role in release. A picture of a single cell experiment has been previously published [16].

There is an ever-growing variety of sources for single cell experiments. Included are both isolated and cultured cells. Among them are neurons from the rat [17], pond snail (*Planorbis corneus*) [18] and common leech (*Hirudo medicinalis*) [19], as

well as neuron analogs, such as the bovine adrenal cell [20], mast [21] and PC12 (pheochromocytoma) cells [22] from rats, and frog oocytes (*Xenopus laevis*) [23]. Additionally, recent studies have examined rat and human pancreatic β-cells [9] and pituitary melanotrophs [24], both of which release peptides/neuropeptides rather than the traditionally studied neurotransmitters.

When a single cell is stimulated, vesicle release occurs rapidly. Each vesicle released produces a current spike at the recording electrode (Fig. 4). The charge passed through the electrode during a single spike reflects the number of molecules which have been oxidized or reduced. Amperometric currents recorded at single cells can be converted to charge via integration with respect to time. Faraday's law allows conversion of charge to number of molecules: $q = (zFN)/N_a$, where q is charge, z is the number of electrons involved in the oxidation or reduction, F is the Faraday constant, N is the number of molecules oxidized or reduced and N_a is the Avogadro number. If an electrode is sufficiently close to the surface of a cell during release, all of the electroactive molecules released from a vesicle will be oxidized

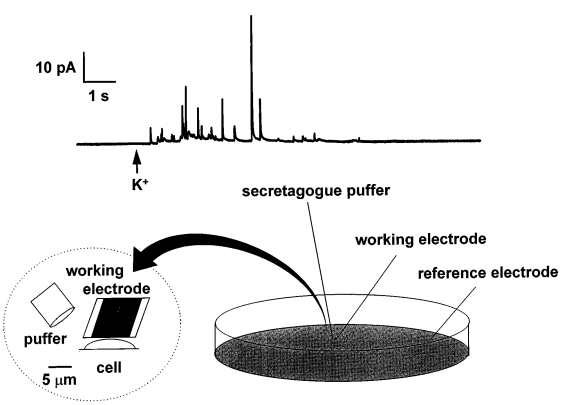

Fig. 4. Amperometric spikes during exocytosis. (Upper panel) 60 mM K^+ was puffed onto a chromaffin cell for 3 s. The amperometric current was recorded at +650 mV vs SSCE. The arrow indicates the beginning of the stimulation. (Lower panel) A cartoon depicts both the macroscale and microscale of single cell experiments. Cultured cells are spread across a petri dish allowing placement of electrodes and puffers with the aid of an inverted microscope (not shown). The inset illustrates the alignment of the working electrode, cell, and puffer, and is approximately to scale.

or reduced. Therefore, the total number of molecules oxidized or reduced during a single spike reflects the number of molecules in a single vesicle. The numbers obtained by amperometric detection coincide well with those using other detection methods [20]. In terms of the simple model of neurotransmission outlined above, single cell analysis allows quantification of the individual release events which are happening at the nerve terminal.

The original measurements made at single cells used bovine adrenal cells, a hormonal analog to neurons [25]. These cells have large vesicles, producing readily measurable current spikes following release. Previous studies using HPLC, enzymology and electron microscopy, separated adrenal cells into at least two categories, those which stored and released norepinephrine and those which stored and released epinephrine. Both amperometry and FSCV were used to monitor release from these cells. At first, neither amperometry nor FSCV was able to distinguish between these molecules. However, two variations of FSCV have since demonstrated successful chemical resolution of norepinephrine and epinephrine. The first used a slower scan rate which greatly diminished the temporal resolution of the data [26,27]. Fortunately, a few years later, a fast-scan technique was developed which was able to distinguish NE and E [28]. This method used new potential limits for the waveform to provide chemical discrimination. The studies revealed a third category of adrenal cells—those which contain both NE and E. Interestingly, it appears that individual secretory vesicles from these adrenal cells contain either NE or E, but not both.

Adrenal cells are easily isolated and remain viable for about a week when incubated. Using a variety of stimulants (secretagogues), including K^+, Ba^{2+}, and digitonin, release events are readily induced. Because these agents are puffed onto the cell, the delay between stimulation and cellular depolarization is poorly defined with secretagogues. An improvement is provided by combination of electrochemical detection with patch clamp technology which allows external control of membrane potential. Using such a system, Neher and co-workers [29] and Fernandez-

Chacon and co-workers [30] were able to characterize the latency between cellular depolarization and release. Additionally, the patch clamp apparatus allows measurements of cellular capacitance, which is proportional to membrane surface area. Any exocytotic event will increase membrane size and membrane capacitance. Thus, two independent signals for exocytotic events are available, the capacitance measurement and the electrochemical signal.

Because the adrenal cell is incubated in a defined media, it is possible to examine how the extracellular environment affects exocytotic release. Several variables have been explored, including temperature [31] and pH [32]. Interestingly, the total free contents of a single chromaffin vesicle are near 1 M. Clearly, association must occur within the vesicle to lower the osmolarity. Additionally, the pH of the contents is quite low, near 5.5. It has been confirmed using electrochemical detection, that the osmotic gradient between the vesicle and the extracellular solution drives release. Additional energy is provided by the pH gradient. When the extracellular osmolarity was raised above that of the vesicle, no release events were observed. However, there were several pre-spike events. These subtle features were first described by Neher and co-workers [29] and represent catecholamine release before the entire vesicle opens. If vesicular opening is driven by osmolarity and pH gradients, it is likely that the vesicle cannot open fully when there is a high external osmolarity. Thus, the vesicles are envisioned to be trapped in a 'flickering' state, in which a fusion pore continually opens and contracts, with the vesicle unable to completely open. Catecholamine seeps through this fusion pore, causing a small amperometric signal, termed the 'foot' of a spike.

Studies in mast cells have revealed similar results [21]. Unlike the adrenal cells, mast cells contain histamine and 5-hydroxytryptamine (5-HT). Again, more sensitive techniques, such as HPLC and capillary electrophoresis were used to confirm the content of the cells. A modified form of fast-scan cyclic voltammetry was developed to distinguish the two analytes. In contrast to the adrenal cells, individual mast cell vesicles contain both 5-HT and histamine which are subsequently co-released.

Since the first reports of electrochemical detection at single adrenal cells [20,25,29,30], several investigators have expanded these investigations to neurons. Included are isolated, cultured and in situ [18,33] neurons. A caveat for all of these studies is the speed at which release occurs at a neuron. Events are so fast, that only amperometry has sufficient temporal resolution to detect them. Thus, the chemical composition of the signal is often assumed rather than determined. For example, using cultured superior cervical ganglions from rats, Zhou and Misler [17] were able to detect release events from individual neurons with amperometric detection. The recorded spikes were very fast (0.5–2 ms duration) and small (2–20 pA) compared to those seen at adrenal cells.

Not all single cell investigations have investigated neurotransmitter release. Recent studies have also focused on peptides/neuropeptides including the detection of insulin [9] and α-melanocyte-stimulating hormone [24]. To use electrochemical detection for peptides, the molecule of interest must contain tyrosine, tryptophan or a thiol. If only thiols are present, carbon fiber electrodes must be modified with a ruthenium oxide coating. Without this catalytic coating, electron transfer rates are too slow to allow peptide detection. Sensitivity is non-linear with respect to the number of electroactive amino acids in the peptide, and the number of electrons per molecule involved in oxidation or reduction needs to be empirically determined. Voltammograms for different peptides can be obtained in vitro for comparison to results in vivo.

3.2. In vitro (brain slices) electrochemical measurements

Electrolytic detection has been used to investigate the neurotransmission of dopamine (DA) [34–36], 5-hydroxytryptamine (5-HT, serotonin) [37,38] and norepinephrine (NE) [39,40] in brain slices. For these studies, thin slices of the brain are continually perfused with oxygenated artificial cerebrospinal fluid and remain viable for several hours. As with single cell experiments, direct ma-

nipulation of the extracellular fluid is possible. Thus, brain slices provide an ideal sample for the study of local effects of drug application. These results are often compared with in vivo studies to remove any systemic effects which might occur following application of a drug. Additionally, unlike systemic administration in vivo, the absolute concentration of the drug present at the slice is known.

Because of the complexity of any whole tissue sample, these studies rely upon electrochemical, pharmaceutical and anatomical evidence to identify and confirm the analyte monitored. The use of stereomicroscopes makes anatomical placement of the microelectrode straightforward for in vitro studies. However, unlike in vivo methodologies, it is usually not possible to directly stimulate cell bodies as they are often not present within the brain slice containing the nerve terminals.

For reasons which are not clear, local electrical stimulation of dopamine release in the slice leads to abnormally high extracellular concentrations, relative to equivalent stimulations in vivo using remote locations. Because of the enhanced release, absolute, but not comparative, analysis of release processes is precluded in the slice. However, the slice offers an ideal system for uptake measurements. With large concentration releases following single-pulse stimulations, it is often easier to make dopamine measurements in the slice as compared to in vivo. A similar problem does not exist for either 5-HT or NE.

FSCV studies in brain slices have recently characterized a newly developed strain of mice which lacks the dopamine transporter (DAT) gene [41]. All three genotypes—wild type, heterozygote and homozygote recessive—were investigated (Fig. 5). The results were intriguing, suggesting a tight regulatory link between the DAT and DA synthesis. Following electrical stimulation, released dopamine remains in the extracellular environment 300 times longer in the DAT knock out animal compared to the wild type. The limiting process for removal of dopamine appears to be diffusion of dopamine from the slice, facilitated by the flow of artificial cerebral fluid over the slice. This result unequivocally demonstrates the importance of the DAT for the removal of dopamine following release. Interestingly, the maximal release of dopamine from the DAT knock out animal was diminished 4-fold. This result helps to explain microdialysis data obtained in vivo which measured only a 5-fold increase in basal levels of dopamine despite the 300-fold decrease in removal of dopamine. Heterozygote mice were also examined. As expected, they had basal levels and removal times of extracellular dopamine about double those of the wild-type animal. To confirm that only dopamine was contributing to the measured signals, NE and 5-HT uptake inhibitors were applied. No change in the release or uptake terms was observed.

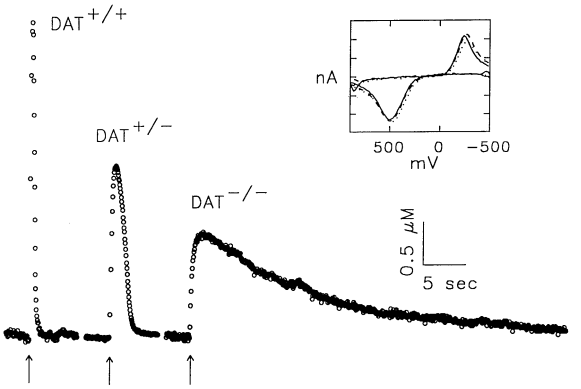

Fig. 5. Monitoring electrically stimulated dopamine release in brain slices. All three genetic variants of the dopamine transporter (DAT): wildtype [DAT(+/+)]; heterozygote [DAT(+/−)]; and homozygote recessive [DAT(−/−)], were studied using fast-scan cyclic voltammetry. The arrow indicates the time of a single bipolar electrical stimulation. The inset shows a background subtracted cyclic voltammogram for each of the three curves. Reprinted by permission from Nature [41] Copyright 1996 Macmillan Magazines Ltd.

Serotonin has been measured by FSCV in two separate manners. First, serotonin was studied using a typical triangle waveform [37]. Although the results were conclusive, it was not possible to monitor uptake events because of the slow temporal response of the electrode. The sluggishness of the electrode was due to adsorption of 5-HT on the electrode, a process avoided by a slight modification of the waveform [42]. With this adjustment, it was possible to study both release and uptake of 5-HT with FSCV [38]. The results mimic those for the other transmitters and have been compared to DA transmission in vivo.

High-speed chronoamperometry studies of NE release in the hippocampus are complicated by 5-HT innervation. To circumvent this problem, investigations have employed oculografts [43] to limit the neurotransmitter content of the sample. By co-grafting locus coeruleus and hippocampal tissue to the eye, it is possible to generate competent noradrenergic innervation of the hippocampus in the absence of 5-HT terminals. For study, the grafts are typically removed and investigated as slices in vitro. Using high-speed chronoamperometry, NE release was measured following local application of K^+. Slices were perfused with nomifensine and phentolamine to further confirm the specificity of the measurement. Unfortunately, electrical stimulation was unable to induce NE release.

3.3. In vivo electrochemical measurements

In vivo electrochemical measurements differ dramatically from those made at a single cell. Because single cell experiments use carbon fiber microelectrodes and piezoelectric positioners, it is possible to place the voltammetric probe within 1 mm of the release site. Additionally, the diffusion path from the site of release to the electrode surface is completely free, allowing detection of virtually every molecule released from a vesicle. Thus, the source for any electrochemical signal is well known for single cell measurements. Contrast this with what occurs in vivo. The size of the electrode, although small, is enormous compared to the size of a typical synapse. For example, the typical striatal dopamine synapse is roughly cylindrical, with a radius of 300 nm and a gap of 15 nm [44]. Therefore, it is not possible to position the electrode directly adjacent to the site of release. Rather, the signals generated at microelectrodes in vivo represent changes in extracellular, i.e. extrasynaptic, neurotransmitter concentration. Interpretation of these changes is complicated by the variety of factors which can influence the neurotransmitter level including release, uptake, diffusion, and metabolism (Fig. 1) [45,46]. Additionally, it is essential to determine the influence of the probe on neurotransmitter levels and account for any perturbation in analysis of results. Once these factors are accounted, it is possible to extrapolate extracellular concentrations to synaptic concentrations, allowing subsequent comparison of in vivo results with those determined at a single cell.

The detection of signals following stimulation demonstrates that neurotransmitters can leave the synapse. Once free of the synapse, diffusion of a molecule cannot occur freely, i.e. along any possible route to the electrode. Rather, the pathway to the electrode is complicated by the cellular structures which impede the molecule's movement, a process described by tortuosity. As the molecule diffuses, it can interact with both receptors and transporters which act as sinks, decreasing the extracellular concentration. Because of these interactions with macromolecules, concentration represents a measure of 'effectiveness' or 'signal strength' for a transmitter in the brain. Normally, receptor binding is considered reversible and uptake irreversible, so depending upon receptor on/off rates, receptor binding may or may not significantly perturb extracellular concentrations of transmitters on the time scale of a voltammetric measurement. A final consideration is enzymatic degradation. However, on the short time scale following stimulation, it is too slow to influence the extracellular concentration of neurotransmitter.

FSCV offers several advantages for in vivo electrochemical measurements. For several years, we have used Nafion-coated, microdisk electrodes to characterize dopamine release throughout the brain. This work has included investigations of the caudate putamen (CP) [47], medial prefrontal cortex (MPFC) [48] and basolateral amygdaloid nucleus (BAN) [49]. These regions vary in their concentration of dopamine terminals, relative to other neurotransmitters, with the CP and NAc predominantly dopaminergic and the MPFC and BAN rich in NE, 5-HT and DA. Confirmation that DA is being monitored is more difficult in the latter two regions, given the presence of other neurotransmitters. Especially, problematic is NE, which has a voltammogram indistinguishable from DA. Therefore, identification of DA has relied upon several other pieces of information from pharmacological and anatomical studies.

First, voltammograms can identify an analyte as a catecholamine, which is a necessary condition for DA detection. Discrimination among catecholamines relies upon pharmacological and anatomical evidence. There are a variety of drugs available which selectively inhibit either release or uptake of dopamine, as there are for the other catecholamines. Additionally, there is extensive anatomical evidence available which describes the expected location of dopaminergic terminals. Within certain anatomical regions, there even exists microheterogeneity, which allows selective positioning of an electrode. Finally, there is the position of the stimulating electrode. The position of the cell bodies which project into a region of the brain is often distinct for each type of neurotransmitter in that region of the brain. For example, it was possible to selectively stimulate dopaminergic neurons in the MPFC despite the presence of noradrenergic terminals nearby based upon the location of the stimulating electrode in the substantia nigra/ventral tegmental area where there are no noradrenergic cell bodies [48].

Considerable effort has been made to reveal the processes which determine the extracellular concentration of dopamine measured at the electrode following electrically stimulated release. A complication which arises when using a Nafion-coated electrode is the increased transit time for dopamine to reach the electrode surface. The delay is attributed to the diffusion coefficient of dopamine in Nafion which is about three orders of magnitude less than in solution. Thus, a time delay will occur between the actual change in extracellular concentration and the signal measured at the electrode. Deconvolution allows the actual signal to be revealed from the measured signal.

Once the temporal effect of Nafion has been removed, it is possible to speculate how the extracellular concentration measured at the electrode relates to events occurring at the synapse and extrasynaptically. We have developed a simple model to describe these processes [46]. It is based upon rate equations which describe release and uptake of dopamine following electrical stimulation. For release, it is assumed that a fixed amount of dopamine emerges from the pool of neurons near the working electrode following each stimulus pulse. For uptake, Michealis–Menten kinetics are assumed. Numerical techniques are used to approximate the expected concentration profile based upon the net rate equation.

The kinetics of dopaminergic transmission in all of the regions listed above (CP, NAc, MPFC, and BAN) were compared, leading to some interesting conclusions about how dopamine might function. For example, it was found that dopamine signals were tightly controlled in the CP and NAc, with rapid uptake preventing significant diffusion of dopamine following release. However, in the MPFC and BAN, dopamine appears to be a neuromodulator rather than a neurotransmitter, given the relative freedom of the molecule to diffuse upon release. Perhaps most significant of all were conclusions drawn from short stimulations in the nucleus accumbens. It appears that dopamine 'acts' extrasynaptically, rather than at the synapse, as is often implied in models of neurotransmission based upon the behavior of acetylcholine at the neuromuscular junction [50].

Amperometry has also been used in vivo to monitor dopamine release [51,52]. Its excellent temporal resolution is sufficient to directly measure kinetic events which can only be extrapolated from FSCV data. For example, using amperometric detection with cylindrical carbon fiber microelectrodes, it was possible to monitor dopamine release following a single electrical stimulation [51]. The kinetics measured in this manner were quite similar to those measured with FSCV [50].

To use amperometry in the brain, however, several precautions and confirmations should be implemented. Anatomical specificity is provided by the locations of both the recording electrode and the stimulating electrode. Pharmaceutical evidence is based upon predicted responses from selective agents, such as uptake blockers and synthesis inhibitors. Additional evidence is provided by local application of known release inhibitors, such as tetrodotoxin and cadmium. Because there is no chemical selectivity, it is essential that amperometric measurements be made only over short time periods. Basal level analysis and long-

term monitoring of neurotransmitters are not possible with amperometry in vivo because of the high concentration of ascorbate and several metabolic products, all of which are electroactive. These analytes vary slowly in vivo, so short-term signals should not be influenced.

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