Analytical and Quantitative in Vivo Monitoring of Brain Neurochemistry by Electrochemical and Imaging Approaches

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ABSTRACT: Quantitative monitoring of brain neurochemistry is aimed at an accurate measurement of chemical basal levels and dynamics defining neuronal activities. Analytical tools must be endowed with high selectivity, sensitivity, and spatiotemporal resolution to tackle this task. On one hand, in vivo electroanalysis combined with miniature electrodes has evolved into a minimally invasive method for probing transient events during neural communication and metabolism. On the other hand, noninvasive imaging techniques have been widely adopted in visualizing the neural structure and processes within a population of neurons in two or three dimensions. This perspective will give a concise review of the inspiring frontiers at the interface of neurochemistry and electrochemistry (microvoltammetry, nanoamperometry, galvanic redox potentiometry and ion transport-based sensing) or imaging (super-resolution single nanotube tracking, deep multiphoton microscopy, and free animal imaging). Potential opportunities with these methods and their combinations for multimodal brain analysis will be discussed, intending to draw a brief picture for future neuroscience research.

1. INTRODUCTION

Brain function or dysfunction shapes our lives through the essential neurotransmission or synaptic transmission process, which is a comprehensive outcome of an interactive, dynamic network of neurochemicals. Establishing a quantitative linkage between neurochemistry and activities of neurons, circuits, intact brain and even the whole living biosystems is the holy grail in neuroscience at the molecular scale. Generally, strategies for building the connection can be invasive and noninvasive analysis, depending on how the neural samples and analytical units/probes are incorporated, of a broad range of chemicals in the brain: ions (H⁺, Na⁺, K⁺, Ca²⁺, Mg²⁺, etc.), gases (O₂, H₂S, NO, etc.), reactive oxygen species, neurotransmitters [dopamine (DA), serotonin, epinephrine, non-epinephrine, glutamate, acetylcholine, etc.], neuromodulators (e.g., ascorbate), energy suppliers (glucose, lactate, pyruvate, ATP, etc.), cell metabolites, peptides, proteins, and nucleic acids. Quite different from widely understood analytical chemistry, this so-called in vivo analysis faces much more challenges in endowing methods with enough selectivity, sensitivity, spatiotemporal resolution, reliability, and neuron compatibility to adapt to the unique complexity of chemical environment in the brain. In the past half century, we have witnessed a fast expansion of this topic into a multidisciplinary field that involves knowledge and techniques of electrochemistry, optical imaging, mass spectrometry, magnetic resonance imaging, and online sampling, and so forth. However, rather than giving a full coverage of all the progress in the field, we are restricted to latest inspiring and prospective research on in vivo quantitation of brain neurochemistry, with a particular focus on electrochemical and imaging approaches.

The task of in vivo quantitation is measuring the basal levels or dynamics of neurochemicals at various neural dimensions, such as single vesicles, neurons, brain regions, neural circuits, brain slices, and intact brains in living animals or humans. This was achieved by time- and labor-consuming tissue/cell sampling, separation, and analysis in early stages, and limitations were the spatiotemporal gaps between the real chemical events in vivo and analytical outputs ex vivo. One solution is the on-line microdialysis-sensing system based on miniaturized push–pull perfusion sampling. Another important direction is the development of the on-site analytical platform for real-time monitoring of neurochemicals in living biosystems. It is typically performed in conditions as close as possible to the real states of biosystems as possible, aiming at extracting accurate and quantitative molecular information encoded in vivo. To achieve this goal, minimally invasive or noninvasive methods applied to unrestricted living entities have emerged in two major intriguing trends: in vivo electroanalysis and imaging. A concise review of frontiers in these aspects will be made in the present perspective. Our purpose is to draw a blueprint for future interdisciplinary collaboration on molecular interpretation of the brain.

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Amines. Thus, sensitivity is crucial for capturing catecholamine transients (below micro molar). A great deal of efforts have been devoted into background subtraction and chemometric data analysis, attempting to dig out signals overwhelmed by non-Faradaic currents, interferences, and noises. In spite of these, Tybrandt et al. described an interesting combination of FSCV and organic electrochemical transistor (OECT) that took advantage of the field effect to amplify FSCV signals and reduce electrical noises. An OECT chip was fabricated to consist of micropatterned gold electrodes as gate electrodes, source, and drain terminals and a microchannel made of poly(3,4-ethylenedioxythiophene):poly(styrenesulfonate) (PEDOT:PSS). The FSCV measurement was conducted by applying the scanning potential to one gate electrode, at which DA was oxidized. The resulting ionic current caused consequential drain current change by modulating the Na+ doping level of the PEDOT:PSS film thought the electrolyte in the channel (Figure 1). Transient measurement of DA concentration down to a few micromoles endowed this hybrid sensing platform with a merely comparable performance in line with the state-of-the-art FSCV equipment, although it has not been tested for implanted applications. Nevertheless, OECT represents a new opportunity for development of in vivo FSCV.

2. IN VIVO ELECTROANALYSIS

2.1. Fast-Scan Cyclic Voltammetry. The history of in vivo electroanalysis was started by Adams and colleagues’ pioneering endeavor on microvoltammetry in the late 1970s. Using an implanted micro-carbon paste electrode, they monitored the levels of catecholamine transmitters in the cerebrospinal fluid in an electrically or chemically stimulated rat brain. This is a milestone in brain neurochemistry as neurotransmission can be directly, continuously, and quickly quantitated. Electroactive substances are electrolyzed at the biased electrode surface to yield faradaic current as the quantitative indicator of concentration. Then the question was asked: how could we clarify identities of coexisting chemical constituents producing currents? The answer was later given by Wightman and colleagues, who reported the breakthrough attempt of in vivo fast scan cyclic voltammetry (FSCV) combined with pyrolytic carbon fiber electrode (CFE) in selective monitoring of fast striatal DA release. The unique advantage of FSCV over ordinary microvoltammetry is the strong resolving power at high potential sweeping speed without gaining large charging background under the aid of CFE. In a subsecond scan, neurochemicals (such as DA and ascorbic acid) undergoing distinguishable electrode kinetics can be resolved to distinct current peaks. Therefore, FSCV is not only useful for selective measurement of neurochemicals of interest but also well-suited for concurrent multicomponent analysis.

FSCV over three decades has advanced from the initial trial on anaesthetized rats into a reliable technique for acute and chronic measurements in freely behaving animals. Being a differential method, FSCV is incapable of determining basal level, while favoring monitoring fluctuations of biogenic amines. Thus, sensitivity is crucial for capturing catecholamine transients (below micro molar). A great deal of efforts have been devoted into background subtraction and chemometric data analysis, attempting to dig out signals overwhelmed by non-Faradaic currents, interferences, and noises. In spite of these, Tybrandt et al. described an interesting combination of FSCV and OECT that took advantage of the field effect to amplify FSCV signals and reduce electrical noises. An OECT chip was fabricated to consist of micropatterned gold electrodes as gate electrodes, source, and drain terminals and a microchannel made of poly(3,4-ethylenedioxythiophene):poly(styrenesulfonate) (PEDOT:PSS). The FSCV measurement was conducted by applying the scanning potential to one gate electrode, at which DA was oxidized. The resulting ionic current caused consequential drain current change by modulating the Na+ doping level of the PEDOT:PSS film thought the electrolyte in the channel (Figure 1). Transient measurement of DA concentration down to a few micromoles endowed this hybrid sensing platform with a merely comparable performance in line with the state-of-the-art FSCV equipment, although it has not been tested for implanted applications. Nevertheless, OECT represents a new opportunity for development of in vivo FSCV.

2.2. Nanoamperometry. Different from CV, amperometry monitors chemical dynamics in the sense of current transients at a constantly held potential. Not relying on the scanning potential waveform to gain resolving power, amperometric sensors require a specifically designed electrode surface/interface to solely permit electrochemical reactions of target molecules according to their intrinsic redox properties. For instance, CFEs are modified with particularly treated carbon nanotubes (CNTs) for selective in vivo amperometric monitoring of ascorbic acid. Because of the ultrasensitivity and ultrafast response speed, amperometry can be performed at very small current scale of a few nanoampere or even picoampere and time scale of millisecond or microsecond. From this perspective it is an attractive tool for probing transient chemical events like exocytosis during synaptic transmission, and in fact Wightman and colleagues gave the early picture of how it worked with chromaffin cells in 1991. As termed single-cell amperometry, it typically requires a working CFE positioned close to or in contact with the membrane of individual cell with a reference electrode in solution. Neurotransmitters released into the extracellular...
space (ECS) from single vesicle are quickly oxidized at the biased sensing probe and produce an anodic current spike. Because the sensing interface and potential are tunable for desired selectivity, we can assess types of neurochemicals packed in a vesicle. A number of vesicular catecholamines like DA, serotonin, epinephrine, and norepinephrine have been verified. Most recently, quantum release of ascorbate from single rat adrenal chromaffin cell upon K⁺ stimulation was confirmed and analyzed by an electrochemically treated CFE at +0.0 V versus Ag/AgCl (Figure 2a), providing the first quantitative description of endogenous ascorbate secretion.¹¹ Number of neurotransmitters during each quantum release is quantitated by the integral charge of corresponding spike, shape of which also provides useful information about release kinetics.¹² For example, in measuring serotonin discharge from single synaptic vesicles of neurons Bruns and Jahn characterized two types of vesicles in terms of release amount and time: small clear vesicles discharging ∼4700 molecules within 260 µs and large dense core vesicles discharging ∼80 000 molecules within 1.3 ms.¹³ By means of single-cell amperometry, Ewing, Amatore, and their co-workers have identified a new partial release mode of exocytosis. As they observed, a small prespike current increase (“prefoot”) and postspike current decrease (“postfoot”) indicate lower neurotransmitter diffusion during opening and closing of the vesicle fusion pore. In some amperometric events, spikes were small and short in pore duration, implying a third “kiss-and-run” exocytosis mode. Unlike the widely accepted “all-or-nothing” full release process, neurotransmission through a partial release mechanism appears to be more modulable, because many factors including lipid composition and membrane proteins may affect the pore opening and closing.
A further step of nanoamperometry into the synaptic cleft (about 20 nm apart) can be realized by nanoelectrodes. By flame etching, Huang, and co-workers fabricated a CFE with a cone-shaped nanotip of 50−200 nm in diameter and 500−2000 nm in length (Figure 2b−e). Quantal norepinephrine spikes were recorded by the nanoCFE inside synapses following high K+ stimulation, over 50% of them featuring complex release sequence that may be explained by the "kiss-and-run" mechanism.18

In order to find more quantitative proof for the partial release model, it is necessary to determine the neurotransmitter amount in vesicles before membrane fusion. Ewing and co-workers showed that single adrenal chromaffin vesicles underwent absorbing onto a microsized carbon disk electrode, sliding over the electrode surface and then membrane rupture to release entire catecholamine content trapped and oxidized by the electrode. This demonstrated the possibility of counting molecules in vesicles by stochastic collisions.19 On the basis of this principle, they established a novel amperometric method termed intracellular vesicle impact electrochemical cytometry (VIEC). In one way, the flame-etched CF nanotip was carefully pushed into the cytoplasmic space without damaging the PC12 cell membrane. Once collided the electrode surface vesicles went through absorbing−sliding−opening to result in a full release of chemical content, 114 500 ± 15 300 molecules. In the other way, the nanotip was placed in contact with the cell membrane to record exocytosis signals upon K+ stimulation and measured released amount, 73 200 ± 5820 molecules. Approximately 64% of vesicular catecholamine neurotransmitters were secreted in single exocytosis events, in good agreement with the open/close mechanism (Figure 3).20 This study also demonstrates that combination of single-cell amperometry and VIEC makes a powerful tool for understanding the dynamic mass transport between cytoplasm and ECS in a real-time manner.

2.3. Galvanic Redox Potentiometry. Compared to voltammetric or amperometric methods, potentiometric methods measure analyte concentration by the interfacial potential difference. Under equilibrium circumstances (zero
network current) according to the Nernst equation, the open-circuit potential (OCP) of the indicating electrode with respect to the reference electrode is determined by relative concentrations of chemicals across the sensing interface. Conventional potentiometric sensors are designed for ion analysis, for example, pH probe.7 Ion-selective membranes enable measuring the transmembrane potential caused by biased transport of target ions. However, in vivo potentiometry has been limited to a narrow range of ionic species due to the lack of suitable permselective membranes for the majority of neurochemicals. Most recently, galvanic redox potentiometry (GRP) was brought up as a new concept of in vivo analysis. GRP is built on a galvanic cell configuration: an anodic indicating electrode (oxidation of analyte, $E_i$) and a cathodic reference electrode (reduction of electron acceptors like $O_2$, $E_R$) connected by a high-impedance voltmeter. In principle, circuit current is infinitesimal enough for reactants to establish thermodynamically determined potentials at equilibria of the heterogeneous electron transfer with electrodes. When $E = E_R - E_i$ is positive, that is, negative Gibbs free energy ($\Delta G = -\text{nFE}$), the whole-cell redox process occurs spontaneously, and the GRP sensor delivers concentration-dependent OCP signals in a self-driven manner.21

Bard and co-workers reported the first demonstration of ultrasensitive potentiometric monitoring of single nanoparticle collision at a microelectrode in 2012,22 bringing OCP-based methods up to the comparable level of nanoamperometry. It is reasonable to foresee real-time measurement of rapid chemical communication between neurons by GRP. In the meantime, we have to admit that it is still at the very beginning stage of its development toward in vivo analysis. Firstly, selectivity is the biggest challenge as the OCP output cannot be resolved, so GRP does not measure the basal level either. The only way out of the riddle is the rational design and modification of the sensing interface (e.g., electrode kinetics, surface binding, and recognition units, etc.) to enhance OCP responses to neurotransmitter transients of interest other than interfering fluctuations. Secondly, steady performance of the reference electrode at constant $E_R$ is key to quantification. Potential disturbance may come from alterations in electron acceptors, pH, and other electroactive species. Microcompartmentalization of the reference electrode in a favorable working environment can avoid unwanted perturbation in $E_R$. Finally, the self-driven nature will aid GRP development in many ways. The most appealing benefit is the improved compatibility with minimally invasive and long-term brain monitoring.

### 2.4. Ion Transport-Based Sensing

Besides electroactive catecholamine neurotransmitters, most neurochemicals are electroinactive and undetectable by direct electrolysis. Enzymatic or nonenzymatic electrocatalysts on the electrode surface are required to overcome the large electron transfer kinetic barrier and generate electron currents. In recent years, rise of ion transport-based sensing methods has introduced a completely different perspective for in vivo analysis of electroinactive molecules.23 Figure 4a illustrates the typical setup of one Ag/AgCl wire (working electrode) placed in a micro or nanopipette with the other (reference electrode) in bulk testing solution. Electrical migration of small ions in electrolyte solution—usually $K^+$ and $Cl^-$—forms ion current, which is readily modulated by the spatial confinement effect of the pipette tip. To be specific, tip geometry, pore size, and surface chemistry will all influence the ion current. Measurements can be performed in two modes. In the CV mode, potentials are repeatedly scanned between +1 and −1 V. Current intensities at two potentials of the same value but opposite signs are compared; if not equal, the phenomenon is named ion current rectification (ICR). ICR was mostly investigated with nanopipettes, asymmetric electrolyte solution or biconical pipettes, until Yu et al. observed micrometer-scale ICR (MICR) at polyelectrolyte brush-modified micropipettes in symmetric electrolyte solution. In the proposed three-layer model, interior surface charge accounted for different current changes at positive and negative potentials.24 An MICR-based biosensor was constructed by neutralizing the polynimidazole layer (positively charged) inside a micropipette with attached ATP aptamers (negatively charged). Cerebral ATP sampled by microdialysis strongly bound the aptamer and caused its dissociation from the inner surface. Exposure of imidazole moieties resulted in an increase of the net surface charge and thus the MICR ratio. A notable achievement with this biosensor is the determination of dialysate concentration of ATP from brain cortex, suggesting that MICR-based methods can measure the basal level of neurochemicals.25

In the amperometric mode, ion current is continuously recorded at a constant potential, which also drives the migration of analytes toward or away from the probing nanopipette tip. In analyzing single particles like biomacromolecules, lipid vesicles or cells, there are three major types of particle–pipette interactions: translocation, collide-and-leave, and collide-and-stay. The former two scenarios produce current spikes by resistive-pulse principle, and the latter one leads to staircase current decrease due to the partial blockage of the tip pore. Up to date, a large body of publications on this topic are about particle translocation kinetics through the nanopore or the nanochannel. In 2016, Li et al. reported a description of single nanoparticle events at the orifice of a nanopipette. In this fundamental study, charged polystyrene particles were employed to unravel the role of particle size, surface property, electric-field force, electroosmotic force, and elastic force in shaping current transients (blip or staircase) upon collision (Figure 4c).26 Although it has not been applied in vivo yet, this method has shown similarities with nanoamperometry in ultrasensitivity and high spatiotemporal resolution. Moreover, it may analyze the nonredox aspects in neurochemistry, such as the membrane composition and electroinactive content of vesicles.

### 3. IN VIVO IMAGING

High-resolution imaging is advantageous in visualizing nanoscale neurochemical dynamics in both qualitative and quantitative ways. As a powerful tool for noninvasive analysis, brain imaging techniques, especially fluorescent microscopy and mass spectrometric imaging, have been extensively reviewed elsewhere12 and are beyond our discussion here. Instead, several latest updates that may represent future research directions will be highlighted in this section.

#### 3.1. Super-Resolution Nanotube Tracking

The microenvironment in the brain is a dynamic community of neurochemicals that varies during physiological and pathological processes. Direct imaging of the structure and organization of the microenvironment in situ has important implications for analyzing brain neurochemistry. Compared to small molecular indicators, fluorescent, or luminescent nanoparticles at moderate diffusion rates are better at completing the task in combination with super-resolution deep tissue localization microscopy. Single-walled CNTs (SWCNTs) stand out as excellent nano-emitters because of their brightness.
in near-infrared luminescent emission, photostability, biocompatibility, and good penetrating ability. Previously super-resolution single SWCNT tracking was proved successful in quantitatively probing molecular motion in cytoplasm and mapping intracellular fluctuation. Godin et al. recently applied this technique to in vivo analysis of ECS in the brain. They injected SWCNTs into live rats before preparation of acute brain slices and collected super-resolution images of SWCNT-intercalated slices. Diffusion and distribution of SWCNTs reflected the structural heterogeneity in forming the microenvironment of ECS. Together with video recording such structural dynamics at nanoscale was monitored by tracking the movement of SWCNTs (Figure 5a). Moreover, the characteristic length-to-diameter ratio of a single nanotube endows it with different diffusion accessibility in response to local environmental fluctuation. Therefore, quantitative information on the interplay of nanotube geometry and physiochemical properties of ECS (e.g., viscosity and molecular crowding) can be extracted.

3.2. Deep Multi-Photon Imaging. Optical imaging of brain tissue over a depth of 30 μm greatly enriches structural and chemical information about neuronal activities. However, tissue scattering limits the signal-to-background ratio (SBR) and thus imaging depth. Although neurons within rat cortex can be monitored by two-photon microscopy, it still involves tissue treatment and optics implantation. Ouzounov et al. lately established a multi-photon imaging platform for noninvasive deep brain imaging at high spatiotemporal resolution. Three-photon microscopy at excitation of 1300 nm enabled capturing activity traces of neurons labeled by genetically encoded calcium indicators. As demonstrated, it exhibited remarkably higher SBR than two-photon microscopy did at the same depth because of the reduced out-of-focus risk at a higher order of nonlinear excitation and scattering at longer-wavelength excitation (Figure 5b). Spontaneous activities of about 150 labeled neurons deep into the hippocampal stratum pyramidale (∼1 mm in depth) were concurrently monitored. This deep multiphoton imaging method offers new opportunities for three-dimensional brain mapping of neurochemical dynamics.

3.3. Brain Imaging at Free Behavioral State. In vivo imaging of neuronal chemical patterns in awake, freely moving animals deciphers the neurochemical basis underlying behaviors. To meet the challenges in sample labeling and spatiotemporal resolution, Zeng, and co-workers engineered a genetically encoded GPCR-activation-based DA probe (GRABDA) by fusing a structure-sensitive green fluorescent protein (cpEGFP) into the membrane DA receptor. Binding of released DA to its receptor triggered protein structure/conformational transitions and then large fluorescence increase of GRABDA. The whole activation process displayed subcellular resolution, fast kinetics (subsecond), high binding affinity (nanomolar) and specificity. Because these probes were expressed in transgenic living animals (fruit flies, zebrafish and mice), flickering fluorescent signals related to rapid
quantum DA release and binding were recorded at free behavioral states (Figure 5c). Moreover, optogenetically elicited nigrostriatal DA release and dynamic mesoaccumbens DA signaling in mice were quantitatively analyzed during Pavlovian conditioning and sexual behaviors. Similar work with structure-sensitive fluorescence was conducted on acetylcholine receptors to analyze acetylcholine-mediated chemical communications. All these successful attempts pave a solid foundation for quantitative imaging of neurochemistry in synaptic events, circuits, and brain function in free animals, especially with the assistance of miniature fast high-resolution microscopy.

4. MULTIMODAL ANALYSIS

Multimodal analysis based on a combination of different sensing methods provides comprehensive information and knowledge to interpret the complex community in the brain. As discussed earlier, single-cell amperometry with intracellular VIEC enables a quantitative comparison of molecular amounts in unfused vesicles and exocytosis to demonstrate the partial release mechanism. Imaging tools can also be correlated to collect comprehensive snapshots of one single event from different perspectives. For example, super-resolution optical microscopy combined with mass spectrometric imaging (e.g., nanoscaled secondary ion mass spectrometry or nanoSIMS) visualize and quantify chemical patterns of isotope-pulsed neurons in the meantime. A detailed description of correlated approaches can be accessed in the review by Phan et al.

Another type of multimodal analysis is the simultaneous monitoring of neurochemicals and neuron spike firings. In early 1990s, Crespi et al. demonstrated that concurrent electrochemical and electrophysiological measurements can be accomplished by a single CFE. This was done in serial mode, that is, chemical and electrical signals alternatively sampled with a very short interval. Wightman and co-workers combined FSCV and cell firing recording by a CFE to investigate the direct role of DA in the reward behavior. In this work, they revealed the coincident DA changes and neuronal firing patterns activated by intracranial self-stimulation in nucleus accumbens. Such a temporal link suggested that their roles may be similar during rewarding and drug self-administration. With the rapid development of microfabrication and micropatterning, arrays of micro or nanoelectrodes can now be integrated onto a miniature substrate for electrical recording parallelized to electroanalysis or optical imaging. Compared to the serial mode, parallel monitoring at multiple sites establishes a spatial link between chemical and electrical patterns in neuron communication and signaling.

5. OUTLOOKS

In conclusion, in vivo electroanalytical and imaging approaches have a promising future in quantitative molecular decoding of neural physiology and pathology. However, we have to admit that accurate deciphering of the correlation between chemical information and live functions of the brain at the in vivo level will remain the biggest challenge in a long period of time. Most of the reported achievements have been obtained with isolated cells, brain slices, and small animals. Longitudinal real-time brain analysis of higher-ordered primates or even humans to track chemical traces in different physiological or pathological states without intervening their activities is still extremely difficult. Following current trends in method development, in vivo brain analysis is stepping into a new era, and its boundaries with a wide variety of research areas are kept blurring. Advanced soft materials possessing good electronic conductivity or transparency and biocompatibility make flexible miniature sensors for implanted and chronic monitoring in free animals or humans. Advanced fabrication techniques enable configuring integrated sensing platforms for high-throughput multidimensional brain screening/mapping. For this purpose, GRP sensor may be an excellent candidate because of its self-driven nature and ease of miniaturization. In addition, microtomy-assisted photoacoustic microscopy (mPAM) is a novel strategy for label-free, automated, noninvasive, and three-dimensional whole brain imaging with high fidelity through simultaneous sensing of nucleic acids, proteins, and lipids. Finally, in vivo brain analysis may be coupled by chemical biological tools (chemical modification, protein engineering, and gene editing, etc.) toward smart regulation of neuron function in response to abnormal chemical fluctuations. This would make brain neurochemistry understandable and controllable as well, being of great significance to early diagnosis and management of neural diseases.
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