World Journal of Pharmacology

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DOI: 10.5497/wjp.v3.i1.1

World J Pharmacol 2014 March 9; 3(1): 1-17
ISSN 2220-3192 (online)

FRONTIER

Implantable (Bio)sensors as new tools for wireless monitoring of brain neurochemistry in real time

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Author contributions: Farina D and Calia G contributed to acetylcholine biosensor and references reorganization; Alvau MD and Puggioni G contributed to glucose and lactate biosensors; Bazzu G and Migheli R contributed to dopamine, norepinephrine, and serotonin microsensors; Rocchitta G contributed to glutamate biosensor, ascorbic acid microsensor, ethanol biosensor, and biotelemetry; Sechi O contributed to oxygen and nitric oxide microsensors and ethanol biosensor; Desole MS and Serra PA contributed to title, abstract, introduction, conclusion, references reorganization, and manuscript overview; Farina D and Alvau MD equally contributed to this study.

Supported by The Regione autonoma della Sardegna (fund P. O. R. SARDEGNA F. S. E. 2007-2013-Obiettivo competitività regionale e occupazione, Asse IV Capitale umano, Linea di Attività l. 3. 1)

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Received: January 15, 2014 Revised: March 3, 2014
Accepted: March 6, 2014
Published online: March 9, 2014

Abstract

Implantable electrochemical microsensors are characterized by high sensitivity, while amperometric biosensors are very selective in virtue of the biological detecting element. Each sensor, specific for every neurochemical species, is a miniaturized high-technology device resulting from the combination of several factors: electrode material, shielding polymers, applied electrochemical technique, and in the case of biosensors, biological sensing material, stabilizers, and entrapping chemical nets. In this paper, we summarize the available technology for the *in vivo* electrochemical monitoring of neurotransmitters (dopamine, norepinephrine, serotonin, acetylcholine, and glutamate), bioenergetic substrates (glucose, lactate, and oxygen), neuromodulators (ascorbic acid and nitric oxide), and exogenous molecules such as ethanol. We also describe the most represented biotelemetric technologies in order to wirelessly transmit the signals of the above-listed neurochemicals. Implantable (Bio)sensors, integrated into miniaturized telemetry systems, represent a new generation of analytical tools that could be used for studying the brain's physiology and pathophysiology and the effects of different drugs (or toxic chemicals such as ethanol) on neurochemical systems.

Key words: Electrochemical microsensors; Amperometric biosensors; Neurotransmitters; Bioenergetic substrates; Wireless biotelemetric technologies

Core tip: Electrochemical microsensors and amperometric biosensors arouse enormous scientific interest because of their low-cost technology and because they guarantee real-time monitoring of changes of the most important brain compounds. In conjunction with miniaturized telemetric devices, the electrochemical sensors, allow the neurochemical monitoring of extracellular space of discrete brain regions in awake, untethered animals for days or weeks. This new scientific approach opens new frontiers for studying the physiological and physiopathological pathways in wild-type animals and in genetic models of the most widespread neurodegenerative diseases.

Farina D, Alvau MD, Puggioni G, Calia G, Bazzu G, Migheli R, Sechi O, Rocchitta G, Desole MS, Serra PA. Implantable (Bio)sensors as new tools for wireless monitoring of brain neurochemistry in real time. *World J Pharmacol* 2014; 3(1): 1-17

Available from: URL: http://www.wjgnet.com/2220-3192/full/v3/i1/1.htm DOI: http://dx.doi.org/10.5497/wjp.v3.i1.1

INTRODUCTION

The identification, observation, and quantification of extracellular biomolecules in the central nervous system (CNS) is a field of growing interest for studying the brain in physiological conditions and for identifying neurochemical changes during neurological diseases. The study of neurochemistry in real time is very important in preclinical (and recently also in clinical) research and for developing new therapeutic strategies for many neuropsychiatric diseases, such as schizophrenia, depression, epilepsy, multiple sclerosis, and neurodegenerative diseases (*i.e.*, Parkinson's and Alzheimer's diseases), and also for neural conditions that deeply influence individual and social behavior such as addiction.

For decades, the extracellular neurochemistry of the CNS has been studied using *in vivo* microdialysis. Microdialysis is a minimally invasive technique suitable for measuring low-molecular-weight compounds in the extracellular compartment of several organs, tissues, or specific brain regions[1]. The microdialysis idea originated in the 1970s with the aim of implanting a hollow dialysis fiber (microdialysis probe) into a tissue for simulating the role of a blood capillary and recovering molecules from the extracellular compartment to highlight their regional changes in concentration[2,3]. When implanted in the brain, the microdialysis probe is perfused with an appropriate Ringer solution (that mimics the composition of the extracellular space fluid) so that neurochemicals are able to diffuse down their concentration gradients out of the probe. The recovered microdialysis samples are analyzed using different analytical methods. The poor temporal resolution and the need to have an available expensive analytical laboratory (for analyzing microdialysis samples) represent the major limitations of this technique.

In recent decades, implantable electrochemical sensors and biosensors have been emerging because of their versatility, their multiple applications, and most of all, their high spatial and temporal resolution[4-6]. In particular, implantable amperometric sensors have been proven to be very sensitive so as to allow the detection of very low concentrations of the studied analytes[5]. The basic idea of implantable electrochemical sensors is to "concentrate" an entire analytical laboratory "on the tip of a pin" without the need of an expensive analytical apparatus or of a dedicated laboratory.

In the past years, despite their high sensitivity, the main limitation for the use of electrochemical sensors was related to their poor selectivity. Recently, the development of new sensing materials and new shielding polymers and, mainly, the introduction of biological elements such as molecular recognition sites have allowed overcoming this limitation in a large part.

Today, each sensor, specific for every neurochemical species, is a miniaturized high-technology device resulting from the combination of several factors: electrode material, shielding polymers, applied electrochemical technique, and in the case of biosensors, biological sensing material, stabilizers, and entrapping chemical nets.

The dimensions of implantable electrochemical sensors vary from a few micrometers (5-10) up to 125 μm (always lower than those of a microdialysis probe, around 220 μm), and their sensing surface can be increased without increasing their invasiveness using new nanomaterials (*i.e.*, carbon nanotubes); this process is often indicated as "nanostructuration" or simply "nano-on-micro". But one of the most exciting perspectives, for future development and applications, is to combine implantable sensors with miniaturized electronic devices in order to transmit neurochemical signals at a distance so that awake animals are allowed to be totally free to move[4-6].

In this study, we highlight the state-of-art of electrochemical microsensors and biosensors, already used in preclinical research for recording neurochemical changes, suitable to be integrated in biotelemetry systems for the wireless monitoring of brain neurochemistry.

IMPLANTABLE (BIO)SENSORS

We have chosen to describe the available technology for the *in vivo* electrochemical monitoring of neurotransmitters (dopamine, norepinephrine, serotonin, acetylcholine, and glutamate), bioenergetic substrates (glucose, lactate, and oxygen), neuromodulators (ascorbic acid and nitric oxide), and exogenous molecules such as ethanol. In the next section, we also describe the most represented biotelemetric technologies to combine with the sensors in order to wirelessly transmit the signals of the above-listed neurochemicals.

Dopamine, Norepinephrine, and Serotonin

Brain neurotransmitters such as the tyrosine derivatives dopamine, norepinephrine and the neuroactive tryptophan derivative serotonin have been implicated in the neurochemistry and physiology of mental diseases and neurological disorders.

Catecholamine biosynthesis is a common pathway from tyrosine[7], where the hydroxylation of tyrosine to L-3,4-dihydroxyphenylalanine by tyrosine hydroxylase is the rate-limiting step. Dopamine, a catechol-like neurotransmitter derived by L-3,4-dihydroxyphenylalanine decarboxylation, is actively involved in reward pathways[8,9] and in cognitive functions[10]. Its metabolism mainly occurs by reaction with monoamine oxidase and catechol-O-methyltransferase with the formation of dihydroxyphenylacetic acid , homovanillic acid, and 3-methoxytyramine. Neuronal death of catecholaminergic cells in the substantia nigra, with a consequent significant reduction of dopamine levels[11] as well as dihydroxyphenylacetic acid, homovanillic acid[12] and

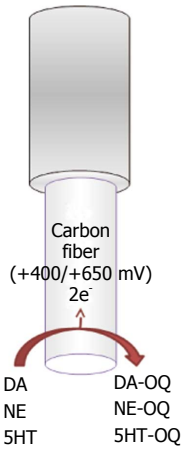

Figure 1 Schematic representation of the carbon-based microsensor used for detecting dopamine, norepinephrine, and 5-hydroxytryptamine in the central nervous system of awake, freely moving animals. DA: Dopamine; NE: Norepinephrine; 5TH: 5-Hydroxytryptamine, serotonin; DA-OQ: DA-derived orthoquinones; NE-OQ: NE-derived orthoquinones; 5HT-OQ: 5HT-derived orthoquinones.

3-methoxytyramine[13] in the striatum is a hallmark in Parkinson's disease[1]. On the other hand, an increase in dopaminergic levels is involved in the etiopathogenesis of schizophrenia[14,15].

Formed by β-hydroxylation of dopamine, norepinephrine plays multiple roles as a hormone and a neurotransmitter. Norepinephrine is involved in directly increasing heart rate, suppressing neuroinflammation[16], and triggering the glycogenolysis and the release of glucose from energy stores[17], and along with serotonin, it is implicated in depression and anxiety disorders[18]. Moreover, the serotonergic system is also implicated in several neuroregulatory processes such as stress, aggression, pain, sleep, appetite, reproduction, circadian rhythm, and cardiovascular and respiratory functions[19].

All of these compounds are electrochemically active, show a similar 2-electron oxidation reaction with similar peak potentials at physiological pH, and can be directly detected by electrochemical oxidation of the molecule[20].

$$\text{DA (Ox)} \rightarrow \text{DA-QUINONE} + 2e^- + 2H^+ \quad (1)$$

$$\text{NE (Ox)} \rightarrow \text{NE-QUINONE} + 2e^- + 2H^+ \quad (2)$$

$$\text{SEROTONIN (Ox)} \rightarrow \text{SEROTONIN-QUINONE} + 2e^- + 2H^+ \quad (3)$$

The electroactive neurotransmitters can be directly detected *in vitro* and *in vivo* using different electrochemical techniques (Figure 1) such as constant potential amperometry (CPA)[21], chronoamperometry[22,23], differential pulse voltammetry (DPV)[24], and fast-scan cyclic voltammetry (FSCV)[8,25-27]. Different microelectrodes for voltammetric recordings in the CNS are available, such as carbon paste microelectrodes, where carbon powder is mixed with silicon oil[10]; epoxy carbon microelectrodes, where epoxy resin is mixed with carbon paste; and carbon fiber, gold, and platinum (Pt) microelectrodes[20].

Along carbon-fiber microelectrodes, FSCV is the most common technique used for dopamine, norepinephrine and serotonin *in vivo* monitoring.

Carbon-fiber microelectrodes (Figure 1) are made by inserting a carbon fiber (outer diameter ranging between 5 and 30 μm, most commonly about 7 μm) into a glass capillary, which is pulled with a pipette puller and sealed by epoxy resin with 25 to 100 μm of the fiber protruding from the glass. The final geometry of the electrode, cylindrical[28] or disk shaped[29], is obtained by cutting or polishing the protruding carbon fiber[30]. Because of their dimension, carbon-fiber microelectrodes minimize distortion caused by ohmic drop, and then, coupled with a minimal tissue damages when implanted into the brain, they are suitable for high-temporal-resolution measurements[28]. In addition, a 7 μm carbon fiber does not stimulate glial reaction[25], in agreement with the evidence that probes that are less than 12 μm in diameter are not encapsulated as demonstrated by previous studies[31]. FSCV is a technique with high resolution and selectivity, where the potential applied to the microsensor is cycled between the reduction and the oxidation peaks of the analyte of interest[20]. For dopamine and norepinephrine recordings, a scan rate in a triangle fashion at 400 V/s is applied. The potential of the carbon-fiber microelectrode is ramped linearly from -400 mV *vs* Ag/AgCl to +1.3 V and back and held at -400 mV between scans[32]. To obtain the 5HT recording, an N-waveform scan rate is used, in which the applied potential is scanned first from 0 mV to +1200 mV then to -600 mV and back to 0 *vs* Ag/AgCl[27]. Typically, the waveform is applied for 10 ms, and voltammetric scans are repeated at 100 ms intervals. During the anodic sweep, the catecholamine (dopamine and/or norepinephrine) and serotonin present at the electrode surface are oxidized into corresponding orthoquinone and then reduced back at the original form during the cathodic sweep. The number of molecules that undergo electrolysis is directly proportional to the measured current[21]. The peak positions during oxidation and the reduction sweep as well as the peak shape can be used to distinguish different analytes[33].

Using fast-scan cyclic voltammetry, dopamine, norepinephrine, and serotonin have been shown a similar oxidation peak at approximately +650 mV *vs* Ag/AgCl[33-35] and a single reduction peak around -200 mV for dopamine and norepinephrine or Wdouble reduction peaks around 0 and -500 mV *vs* Ag/AgCl for serotonin[27].

Because they are virtually identical, voltammograms alone cannot be used to distinguish dopamine and norepinephrine[36], but histology and pharmacology, such as the use of dopamine drugs (raclopride, GBR 12909), can aid in this distinction even in simultaneous measurements with FSCV[37]. Ascorbic acid is the main electroactive interference molecule in the extracellular fluid (ECF) of the brain for electrochemical measurements. Ascorbic acid is 10^4-10^6 times higher than the concentrations of catecholamines in the ECF of the brain, and its concentration is approximately 0.5 mmol/L[37,38]. The carbon-fiber microsensor selectivity for catecholamines can be enhanced

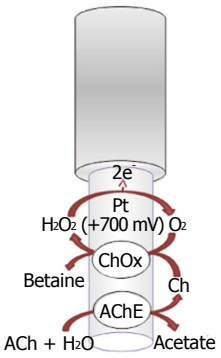

Figure 2 Schematic representation of the platinum-based biosensor used for detecting acetylcholine in the brain of awake, freely moving animals. ACh: Acetylcholine; Ch: Choline; ChOx: Choline oxidase; AChE: Acetylcholinesterase.

by applying on fibers a negatively charged resin (Nafion) able to concentrate cations such as dopamine on the active surface of the sensor and, at the same time, to repel anions such as ascorbic acid and dihydroxyphenylacetic acid[22,39].

Although carbon-fiber microelectrodes are the most used sensors for dopamine and norepinephrine for *in vivo* recording, new strategies are developed to monitor catecholamines real time in the brain.

As recently suggested by Njagi *et al*[40], an amperometric biosensor can be fabricated deposing an enzyme, such as tyrosinase, onto the surface of a carbon-fiber electrode. The enzyme immobilized in a biocompatible matrix and with a final diameter of about 100 μm provides an alternative to FSCV for *in vivo* monitoring of dopamine[40].

Acetylcholine

The neurotransmitter acetylcholine and its metabolite choline play a critical role in various functions of the CNS[41]. The concentration of acetylcholine in the ECF of the brain is 0.1-6 nmol/L[42]; the abnormalities in their concentrations are related to several neural diseases[43]. In particular, it is involved in learning and memory formation[44], in the development and maintenance of addiction[45], and in neurodegenerative disorders such as Alzheimer's disease[46] and Parkinson's disease[47,48]; dysregulation of cholinergic transmission is correlated to cognitive alterations such as those manifested in Alzheimer's disease[49]. Furthermore, organophosphorus (OP) and carbamate pesticides and neurotoxic compounds are capable to inhibit the acetylcholinesterase enzyme (AChE), which is responsible of the hydrolysis of acetylcholine[50].

Therefore, the *in vivo* determination of acetylcholine and choline is important because a rapid and an effective method for simultaneous determination of levels of acetylcholine and choline is needed for the characterization of cholinergic transmission in normal and pathological physiology[51,52]. The most common methods developed for the simultaneous determination of acetylcholine and choline require a conversion into more easily detectable compounds[52].

A lot of strategies have been used to obtain selective detection for acetylcholine and choline with biosensors. Among all acetylcholinesterase-based biosensors, amperometric acetylcholinesterase/choline oxidase (ChOx) biosensor is especially performing because of its potential high sensitivity, reproducibility, and excellent selectivity for *in vivo* simultaneous determination of neurotransmitters; these devices are usable for *in situ* determination of choline and acetylcholine and have been implanted in rat brain[51]. The working mechanism of acetylcholinesterase (Figure 2) is based on the following biochemical reaction[53]:

$$ACh + H_2O \rightarrow Ch + acetate \quad (4)$$

While the choline, in the presence of oxygen, is oxidized by choline oxidase, forming hydrogen peroxide (H_2O_2), which can be easily oxidized onto electrode surface:

$$Ch + H_2O + 2O_2 \rightarrow betaine + 2H_2O_2 \quad (5)$$

The oxidation current of hydrogen peroxide can be used for the evaluation of acetylcholine, choline, and acetylcholinesterase activity. Acetylcholine signal is attenuated by acetylcholinesterase inhibitors such as neostigmine or physostigmine[54,55]. The enzymes acetylcholinesterase and choline oxidase are immobilized on the solid electrode surface such as platinum-iridium (Pt/Ir)[51,56] (Figure 2) or carbon fibers[57]. In order to prevent signal of interferents, different shielding strategies are currently used different. For example, ascorbate oxidase (AAO) is used to minimize interference from ascorbic acid, which is present in relatively high concentrations in the brain ECF[58]; polymeric films are also used onto the sensor surface that limit the access of potential interferences due to electrostatic repulsion (*e.g.*, Nafion) and nonconducting polymers [*e.g.*, poly-(phenylenediamines) (PPD)] that restrict the permeability of small organic molecules (*e.g.*, major interferences ascorbate and urate) while retaining a high permeability to small species such as hydrogen peroxide[59]. The acetylcholinesterase/choline oxidase layer is trapped onto the surface electrode by the cross-linking of amino groups of the enzymes with glutaraldehyde[51]. Moreover, the enzyme layer also includes bovine serum albumin (BSA) that provides stabilization of the enzyme activity in the immobilized state[51].

Hence, the amperometric sensors for acetylcholine and choline are successfully applied and provide a useful tool to analyze basic mechanisms of cholinergic physiology in normal and pathological conditions and those involved in the activity of pharmacological cholinergic drugs.

Glutamate and ascorbic acid

Even if glutamate is a nonessential amino acid, it has been shown to be the most abundant in the brain. As fully described, glutamate represents the most important excitatory neurotransmitter. In plasma, glutamate concentrations reach 50-100 μmol/L while in the whole brain, they are 10-12 mmol/L, but we must take into account that glutamate reaches only 0.5-2.0 μmol/L in ECFs[60].

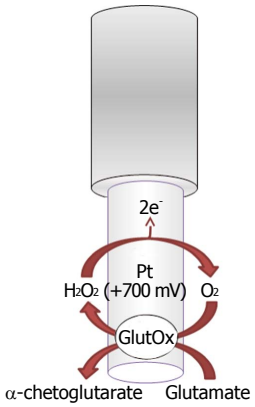

Figure 3 Scheme of glutamate biosensor. The transducer is made of a platinum (Pt) wire that immobilizes the glutamate oxidase (GluOx) enzyme that selectively transforms glutamate in alpha-ketoglutarate, producing H_2O_2 that is then oxidized on the Pt surface.

Glutamate is well known to be involved in most phases of normal brain functions such as memory and learning, cognition, cell migration, differentiation, and death; but at the same time, it is known to play important roles as a highly toxic endogenous excitotoxin[61]. Recently, some authors have highlighted its involvement not only in the development of the CNS, particularly related to neuronal survival, growth, and differentiation, but also in the development of several circuits[62]. In this regard, for example, it has been widely shown that low glutamate levels during neurogenesis may have a key role in the development of schizophrenia[63], and high glutamate levels can also interfere with astroglial proliferation and neuronal differentiation[61]. Glutamate has been of particular importance because of its possible involvement in neurodegenerative diseases such as amyotrophic lateral sclerosis, multiple sclerosis, Parkinson's disease, and others. In fact, the chronic overexcitation of neurons, stimulated by glutamate, is a newer concept that has linked glutamate excitotoxicity to neurodegeneration in amyotrophic lateral sclerosis, Huntington's disease, Parkinson's disease, and Alzheimer's dementia[64].

The importance of glutamate has generated a strong interest in the development of several tools for the detection of this amino acid. Different methods have been developed to determine glutamate, including optical methods, patch clamp, and microdialysis[65], but also including fluorometric, chromatographic, or spectrophotometric techniques, which, however, have some intrinsic limitations, such as being time-consuming, requiring pretreatment of the sample, being labor intensive, and requiring skilled handling. Nowadays, electrochemical methods are considered as one of the most promising approaches because of easiness, high spatial resolution, high sensitivity, and specificity[66]. From the neurochemical point of view, a wide range of amperometric biosensor designs, based mainly on glutamate oxidase enzyme loading [GluOx; molecular weight, 140 kDa; solution Michaelis constant (KM), 0.21 mmol/L in neutral buffer; pI, 6.2], have been developed[67-75].

The aim of monitoring brain glutamate using amperometric biosensors, however, is very challenging, mainly because the baseline ECF concentration of glutamate is estimated to be $\leqslant 5$ μmol/L[76-103].

Glutamate oxidase-based biosensors (Figure 3) exploit the capability of the oxidase to selectively convert L-glutamate as follows:

$$\text{L-glutamate} + H_2O + \text{GluOx/FAD} \rightarrow \alpha\text{-ketoglutarate} + NH_3 + \text{GluOx/FADH}_2 \quad (6)$$

$$\text{GluOx/FADH}_2 + O_2 \rightarrow \text{GluOx/FAD} + H_2O_2 \quad (7)$$

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^- \quad (8)$$

The byproduct hydrogen peroxide is then oxidized, on the transducer surface, by applying a positive potential generating a current flow directly proportional to the glutamate concentrations.

Pt generally is the electrode material of choice for electrooxidation of hydrogen peroxide[77,78]. Various strategies are as well realized in order to shield the biosensor from electroactive interfering substances that usually occur in ECF: first of all, ascorbic acid, through the electrochemical deposition of polymers[68-74]; the use of anionic substances such as Nafion[68,70,79]; or the coimmobilization of the ascorbate oxidase enzyme[75].

The amperometric biosensors have been proven to be interesting devices for *in vivo* measurement of glutamate concentrations and also for their response time, which has been estimated to be about a few seconds[73,74], making these biosensors suitable for the study of the rapid changes in the concentrations of glutamate both in physiological conditions or during pharmacological treatments.

Ascorbic acid is a water-soluble vitamin. It is widely known for its role as an antioxidant, but it is as much recognized as a cofactor in several enzymatic reactions, including those concerning the synthesis of catecholamines, carnitine, or cholesterol[80].

Because humans are lacking the enzyme L-gulono-1,4-lactone oxidase, they cannot synthesize ascorbate, so they, therefore, have efficient machineries for both absorption and recycling of this vitamin[81]. Among them is the transporter sodium-dependent vitamin C transporter-1 (SVCT1) involved in the body homeostasis of ascorbic acid, and the transporter SVCT2 that is necessary for the defense of active cells against oxidative stress[82]. Even the ubiquitous GLUT-type glutamate transporters play a key role in the homeostasis of this vitamin inasmuch as they are involved in the uptake of dehydroascorbate, the oxidized form of ascorbate, in order to be recycled to ascorbate[83].

In the CNS, ascorbic acid is an essential micronutrient, and although the entire brain concentrations are between 1 and 2 mmol/L, the neuronal concentrations have been evaluated to be as high as 10 mmol/L, whereas concentrations in glial cells are about 1 mmol/L[84,85]. At the same time, the ascorbate concentrations present in brain ECF have been estimated to comprise between 200 and 400 μmol/L[81].

Those findings suggest not only that ascorbate has a

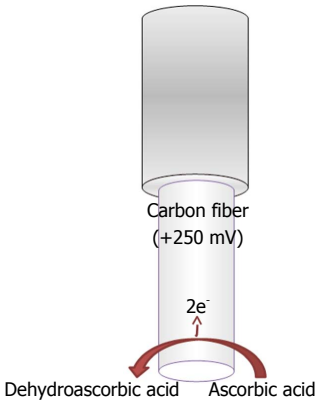

Figure 4 Scheme of AA sensor used in constant potential amperometry. In this representation, the transducer is made of a carbon fiber. The AA is oxidized by applying mild potentials (+250 mV or less) needed for oxidizing the AA to dehydroascorbic acid.

significant role in normal neuronal physiology but also that, given the structural characteristics as an electron donor and free-radical scavenger, it has assumed its role as a neuroprotective molecule and as an important component of the neuronal antioxidant pool[81].

Neurons and glia are able to interact with each other in order to conserve CNS ascorbate, using the mechanism of heteroexchange in which ascorbate release is related principally to glutamate uptake[86,87].

Ascorbic acid is easily oxidized in the following manner (Figure 4)

$$\text{L-ascorbic acid} \rightarrow \text{Dehydroascorbic acid} + 2e^- + 2H^+ \quad (9)$$

by applying a mild anodic potential[4] at the transducer surface (Figure 4), when a constant potential is applied, and generating a current flow directly proportional to the ascorbate concentrations.

For ascorbate *in vivo* monitoring, the transducer is typically made of composite materials of carbon such as carbon paste[87,88] or fibers[89] and multiwalled carbon nanotube (MWNT)-modified carbon fibers[90].

The transducer surface is sometimes modified for excluding electroactive interfering species such as positive catecholamines, so the electrode modification is carried out by the deposition of overoxidized poly (1,2-phenylenediamine)[89].

Cyclic voltammetry (CV)[89,90], square-wave voltammetry[89], and differential pulse voltammetry[91] have been used for *in vivo* measurements of ascorbic acid in the brain of animal models. The latter methods have been proven to be the most sensitive for sensing and biosensing because they change the potential pulsing from one potential to another in a relatively short range of time, different to what happens for the CV where the potential is constantly modified in a linear way[92].

Constant potential voltammetric techniques have also been used for *in vivo* monitoring of ascorbic acid in the brain by applying mild positive potentials such as +120 mV *vs* Ag/AgCl, when this is the implanted reference electrode (RE)[4], or +250 mV when the implanted RE is Ag^+[93].

All the applied techniques have confirmed what was found with other methods that the ascorbate concentrations present in neuronal extracellular spaces are close to 500 μmol/L, emphasizing the reliability and specificity of the reading of the ascorbic acid sensors.

Glucose and lactate

Glucose, a main nutrient in the brain[94], is the most important factor for its energetic metabolism[95-98] and is actively involved in ATP synthesis; it is an important modulator of memory in multiple tasks and improves memory in patients with Alzheimer's disease and Down's syndrome[99,100].

Lactate is another important molecule involved in brain energetic metabolism as energetic substrate for neurons[96] or product of glycolysis under anaerobic condition[94,97].

For a long time, lactate production in the brain was viewed as a lack of oxygen, as the lack of an aerobic oxidation process, or as a mismatch between glycolytic and oxidative rates, but it has recently been identified as an alternative food to glucose[97,100,101].

Contemporary studies in the amount of glucose and lactate in the brain are significant both in physiological conditions and in the presence of disease[102-104].

The recognition and quantification levels of glucose and lactate are possible by using innovative devices such as biosensors constituted by an electric transducer and a biological component such as enzymes; for example, glucose oxidase (GOx), L-lactate oxidase (LOx), or L-lactate dehydrogenase (LDH) is commonly used in the design, respectively, of glucose and lactate amperometric biosensors and their exploiting simple enzymatic reactions and relatively easy sensor design configuration[105]. In particular, amperometric methods have been widely used in glucose and lactate sensing. The biochemical reactions, in presence of oxygen, occurring at glucose and lactate biosensors are as follows[5,106,107]:

$$\beta\text{-D-Glucose} + FAD^+\text{-GOx} \rightarrow \text{D-Glucono-}\delta\text{-Lactone} + FADH_2\text{-GOx} \quad (10)$$

$$FADH_2\text{-GOx} + O_2 \rightarrow FAD^+\text{-GOx} + H_2O_2$$

$$\text{L-Lactate} + FAD^+\text{-LOx} \rightarrow \text{Pyruvate} + FADH_2\text{-LOx} \quad (11)$$

$$LOx + O_2 \rightarrow FAD^+\text{-LOx} + H_2O_2$$

$$\text{L-Lactate} + NAD^+\text{-LDH} \rightarrow \text{Pyruvate} + \text{NADH-LDH} \quad (12)$$

In the electrochemical biosensor (Figures 5 and 6), the hydrogen peroxide byproduct from oxidase enzymes is directly proportional to the quantity of substrate glucose or lactate transformed by the enzymes as shown below in equation (8)[4].

Many studies of neuronal applying biosensors in experimental models *in vivo* are present in literature[108]. These studies show different types of biosensor designs, made with several transducer materials. Biosensors are mainly composed of noble metals, such as gold and/or

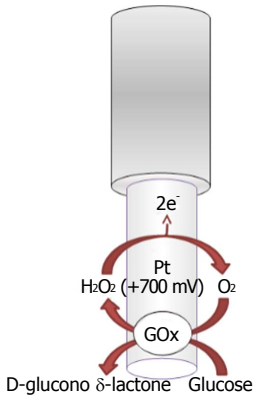

Figure 5 Schematic representation of the platinum-based biosensor used for detecting extracellular glucose in the central nervous system of freely moving animals. The immobilized glucose oxidase (GOx) selectively transforms glucose in D-gloconolactone in the presence of molecular O_2 and generates H_2O_2 that is promptly oxidized on platinum surface.

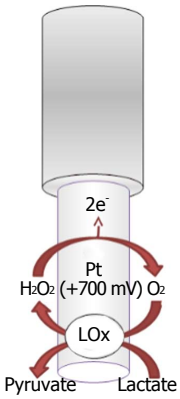

Figure 6 Schematic representation of the platinum-based biosensors for detecting extracellular lactate in the central nervous system of awake animals. In the presence of O_2, the immobilized enzyme [lactate oxidase (LOx)] selectively converts the substrate (lactate) in the corresponding product (pyruvate) and generates H_2O_2 that is oxidized on the Pt surface.

Pt, although recently, other systems use conductive carbon based materials.

A new approach for the simultaneous detection of brain glucose and lactate in real time is reached by the use of a biotelemetric device fixed on the head of the animal[109-111].

In a previous study[6], O-phenylenediamine (OPD) monomers were electrodeposited onto a Pt/Ir cylinder electrode (diameter, 125 μm) surface. The next step was to immobilize GOx, stabilized with polyethylenimine (PEI), by immersing the transducer in the BSA solution and after in the glutaraldehyde solution (GTA). The lactate biosensor was initially made in the same way by changing the oxidase enzyme, but substituting the BSA/GTA with a final layer of polyurethane (PU)[6] for increasing the linear region. CPA was used, fixing the applied potential for hydrogen peroxide oxidation at +700 mV *vs* Ag/AgCl RE.

There are numerous problems with this approach because it is necessary to apply a high potential to detect hydrogen peroxide (+700 mV)[112,113] and the concentration of oxygen can change in the region in which the biosensor is implanted and the resulting current is not directly correlated with the extracellular concentrations of lactate[113-115].

Furthermore, the presence of interfering electroactive species in the tissues and the reactions of biopolymerization are needed to be considered[116,117]. In the nineties, to solve these problems, Karyakin proposed to modify the transduction element using carbon compounds coated with a thin film of Prussian blue (PB), Fe4 [Fe(CN)6]3[113,114,118-121].

After the introduction of PB in the field of biosensors were formulated different materials as supports and methodologies of deposition to improve its electrocatalytic properties and stability[122]. In recent years, some research groups have worked on glucose and lactate microbiosensors based on PB electrodes made of carbon fiber (CFE) modified to detect enzyme-generated hydrogen peroxide low applied potential (0 mV).

Afterward, the enzyme stabilizer PEI was added to improve the performance of the enzyme[122], and GOx and LOx were subsequently immobilized. In order to avoid signal of interferents, OPD was electrodeposited[122]. For the first time, a glucose and lactate microbiosensor, based on PB-modified CFE, is able to detect physiological changes in molecular levels at a low applied potential in the CNS[123].

Moreover, the ultrasmall biosensor size is apposite for *in vivo* neuroscience studies. In contrast, the first generation of microbiosensor transducers based on noble metals have high dimensions (diameter, approximately 100 μm) even if they have been used successfully over the last few decades for the monitoring of neurochemical species[116]. Consequently, the use of carbon-fiber microbiosensors (diameter, approximately 10 μm), modified with PB, seems to be more suitable for use in these studies because it reduces brain damage during insertion[124] and provides an even higher temporal resolution, allowing the real-time correlation with animal behavior[125].

Oxygen and nitric oxide

Oxygen and endogenous nitric oxide are gaseous molecules playing a pivotal role in mediating important biological processes yet are involved in very distinct aspects of organism physiology. Oxygen is indispensable for animal life; an adequate tissue oxygen content, delivered by hemoglobin through the bloodstream, is fundamental to supply cellular metabolic demands, as oxygen is involved in energy production as well as in aerobic cellular metabolism[126].

In contrast, an insufficient oxygen concentration in tissues leads to hypoxia, a severe altered condition in which low oxygen availability prevents aerobic metabolism and oxidative phosphorylation in the cell, yielding to impoverishment of high-energy compounds such as ATP and, lastly, inducing cellular dysfunction and death[127,128].

Though oxygen is a crucial substrate for cellular

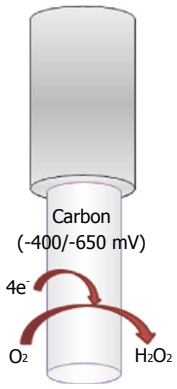

Figure 7 Schematic representation of the carbon-based sensor used for detecting the molecular O_2 dissolved in the extracellular space of the brain of freely moving animals. The O_2 is reduced on the carbon surface at low potentials and converted to water in a one- or two-step reaction (see text).

functions, it also provokes damage because of the toxicity of oxygen-derived reactive species (ROS), such as hydrogen peroxide, singlet oxygen, hydroxyl radicals, and superoxide anion[129]. ROS free radicals attack lipids, proteins, DNA, and RNA and expose cells to oxidative stress, which has been demonstrated to be involved in the pathogenesis of several neurodegenerative diseases[129,130].

Endogenous nitric oxide is a gaseous signaling molecule released in low concentration (tens of nanomoles to low micromoles), characterized by possessing a lifetime of a few seconds[131], as nitric oxide is a highly reactive free-radical species. Nitric oxide production mainly involves the enzymes NO-synthases, which catalyze nitric oxide formation as a byproduct of the reduction of the amino acid L-arginine into l-citrulline[132,133]. Nitric oxide acts as a transitory paracrine and autocrine signaling molecule, by activating the soluble guanylyl cyclase, increasing cellular cyclic guanosine monophosphate (c)[134].

Since its discovery in 1987[135-137], when first nitric oxide was recognized as being involved in the physiological actions of endothelium-derived relaxing factor, mediating vasodilatation, the knowledge of the important role that nitric oxide plays in physiopathology and pharmacology exponentially increased. In fact, further studies revealed how nitric oxide actions are implicated in the cardiovascular system, in the immune response[138], as well as in the nervous systems, mediating neurotransmission[131,139]. Furthermore, nitric oxide is a mediator of both antitumor and antimicrobial activities[140].

Otherwise, the disruption of nitric oxide production seems to be involved in diseases such as atherosclerosis[141], hypertension, cerebral and coronary vasospasm, and ischemia-reperfusion injury. In fact, nitric oxide is attacked by ROS, specifically by superoxide anion, forming peroxynitrite, which generates further reactive nitrogen species (RNS) such as nitrogen dioxide and dinitrogen trioxide. Like ROS, RNS damage lipids, proteins, and other macromolecules, thus also contributing to the onset of diabetes and neurodegenerative diseases[141-143].

The detection of oxygen and nitric oxide tension in the brain has been studied *in vivo*, providing critical information about the physiopathology and pharmacological implications of these molecules.

A wide variety of O_2-sensitive microsensors have been developed. Electrochemical devices exploiting amperometric techniques of detection, such as CPA, differential-pulse amperometry (DPA), CV, and fast-scan voltammetry (FCV), allow the reliable direct reduction of oxygen. Carbon paste and noble metal transducers are the most commonly diffused. Reactions involved in the electrochemical reduction of oxygen at the electrode's surface can occur *via* two mechanisms: a single-step reaction yields to detectable intermediates (Figure 7):

$$\text{oxygen} + 4H^+ + 4e^- \rightarrow 2H_2O \quad (13)$$

In the second mechanism, two-step O_2 reduction forms H_2O_2 as measurable intermediate:

$$\text{oxygen} + 2H^+ + 2e^- \rightarrow H_2O_2 \quad (14)$$

$$H_2O_2 + 2H^+ + 2e^- \rightarrow 2H_2O \quad (15)$$

Changes after physiological stimulations or pharmacological treatments were recorded in the extracellular space of the striatum, by using optic microfibers, assessing that oxygen concentration is about 50 μmol/L[143].

Electrochemical oxygen microelectrodes using CPA at a noble metal transducer bare, such as gold or Pt, allowed the long-term monitoring of oxygen subcutaneous and venous dynamics[144,145].

Nevertheless, several groups preferred to use carbon-paste electrodes (CPEs) because of their longer *in vivo* stability, less surface fouling[146], and quite easy manufacture[147] (Figure 7). Venton *et al*[148] used the FCV technique in a study in which dissolved oxygen was measured in the rat caudate-putamen, by using 5 μm Nafion-coated carbon fibers with a subsecond time resolution. FCV was used also in a study that targeted oxygen levels in the striatum of primates during reward delivery. In this case, the diameter of the carbon fibers ranged from 12 to 33 μm[149].

Lowry *et al*[101,150,151] largely used carbon paste-based miniaturized electrodes in an experimental session in which the effects of anesthesia were studied *in vivo*, as well as the effects of hypoxia and hyperoxia on brain energy metabolism in the striatum[147-149]. Changes in oxygen at CPEs were usually monitored by using the DPA technique[151,152]. Two equally sized cathodic pulses were applied: the first from a resting potential at -150 to -350 mV, corresponding to the foot of the reduction wave for oxygen, and the second, which corresponds to the peak of the reduction wave, from -350 to -550 mV.

In addition, oxygen microsensors were used by Finnerty *et al*[153] in real-time monitoring of oxygen levels in an animal model of schizophrenia, coupled with the use of a glucose biosensor and an nitric oxide microsensor. Oxygen reduction at CPEs has been widely detected also *via* CPA[152]. For example, by applying a constant cathodic potential of -650 mV *vs* a saturated calomel RE, oxygen reduction was recorded in real time in the hippocampus of freely moving rats[115].

Furthermore, CPEs of 200 μm in diameter were implanted in the dorsal and the ventral hippocampus of rats

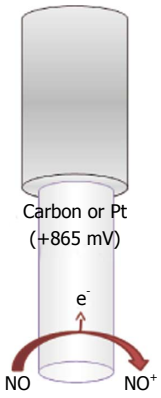

Figure 8 Schematic representation of the more widely used sensor for detecting NO in the brain of freely moving animals. The NO is directly oxidized on a carbon (or platinum) surface to NO^+. This sensor is particularly sensitive to electroactive interferences in virtue of the very high oxidation potentials.

to investigate spatial processing and anxiety. Even in this case, the applied potential was -650 mV *vs* a silver wire REF[154]. The CPA technique was also used by Bazzu *et al*[110] to monitor striatal oxygen levels in a telemetric *in vivo* study. Working electrodes, consisting of miniaturized conical-shaped epoxy-carbon electrodes (180 μm), allowed oxygen detection by fixing the reduction potential at -400 mV *vs* Ag/AgCl REF.

Recently, oxygen amperometry was applied to a behavioral study of reward processing in the rat nucleus accumbens. CPEs (200 μm in diameter) were used by applying a constant potential of -650 mV *vs* a silver wire REF to reduce oxygen. Data showed similar results to those obtained in human fMRI studies, confirming how oxygen amperometry is a powerful technique for the measurement of brain function[155].

In the attempts of monitoring the concentration of the unstable nitric oxide molecule *in vivo* and to test nitric oxide donor drugs, several microsensors have been developed since the 1990s[156]. The majority exploits electrochemical amperometric techniques to directly detect nitric oxide. Commonly, an oxidant potential is applied (higher than +850 mV *vs* Ag/AgCl), in view of the fact that nitric oxide and oxygen reduction potential are very close, so oxygen interferes with nitric oxide measurement (at nitric oxide-reducing potentials) (Figure 8).

Basically, a double reaction occurs at the transducer's face, usually carbon fiber or noble metals[157-161], involving the formation of NO^+, which is further converted into nitrite (Figure 8):

$$NO - e^- \rightarrow NO^+ \quad (16)$$

$$NO^+ + OH^- \rightarrow HNO_2 \rightarrow H^+ + NO_2^- \quad (17)$$

Otherwise, metalloporphyrin-modified sensors[162-164] are also largely used:

$$Fe(II) + NO \rightarrow Fe(II) - NO \quad (18)$$

$$Fe(II) - NO + H^+ + e^- \rightarrow Fe(II) - HNO \quad (19)$$

$$Fe(II) - HNO + 2H^+ + 2\,e^- \rightarrow Fe(II) + H_2NOH \quad (20)$$

Because of the enormous interest kindled by the wide range of actions of nitric oxide, several *in vivo* experiments were conducted to monitor nitric oxide release on different tissues[165-168]. Friedemann *et al*[169] developed an electrochemical electrode using carbon fiber as a transducer, coated with Nafion and further electropolymerized with OPD. Nitric oxide was quantified amperometrically using differential pulse voltammetry[169].

Wu *et al*[170,171] research group conducted several experiments in which physiological nitric oxide actions on a cat' s brain were investigated. Nitric oxide concentration was measured in real-time using voltammetry techniques, implanting Nafion-/porphyrin-/OPD-coated carbon-fiber electrodes. A highly sensitive and selective NO electrode was used to measure the nitric oxide concentration in a rat hippocampus[172]. In addition, an electrochemical nitric oxide microbiosensor based on cytochrome C, immobilized onto a functionalized conducting polymer layer, was implanted in the striatum. Nafion was used for its shielding properties toward interference electroactive molecules present in the brain, chiefly ascorbic acid[173]. Brown *et al*[174] and Finnerty *et al*[175] obtained a simple and useful design by modifying a Pt sensor with multicoated Nafion layers. This electrochemical sensor was successfully implanted in the striatum of freely moving rats, allowing the real-time nitric oxide at Nafion-coated Pt. Santos *et al*[176] recently developed an electrochemical biomimetic sensor based on nanocomposite hemin-based microelectrode, measuring exogenous NO in the rat hippocampus *in vivo* using CV.

Ethanol

In the last decades, ethanol has become the most widespread psychotropic toxic substance in Western countries because it is widely legally accepted and also because it is available at a low cost. Acute, subacute and chronic exposure to ethanol may have important effects on the CNS, therefore it becomes significative to monitor ethanol kinetic and its effects on the brain using the most appropriate techniques[177]. The main effects of ethanol consumption cause significant effects on the CNS, principally enhancing the action of the neurotransmitter GABA and generating disinhibition, ataxia, and sedation[178]. Subchronic exposure to ethanol enhances the dopamine neurotransmission in the mesolimbic system[179,180] and increases dopamine levels in the nucleus accumbens[181], playing an important role as a "rewarding" molecule[182-184].

Recently, implantable electrochemical biosensors have been developed for monitoring the real-time changes of ethanol concentrations in the brain ECFs of freely moving animals (Figure 9). As previously described for other implantable biosensors, the ethanol biosensor exploits the presence of an enzyme, the alcohol oxidase, to selectively quantify ethanol using the production of a directly oxidizable byproduct (hydrogen peroxide), electrochemically detectable on the surface of a Pt transducer[185,186]. The main characteristic of this biosensor is its capability of monitoring ethanol changes second by second and over

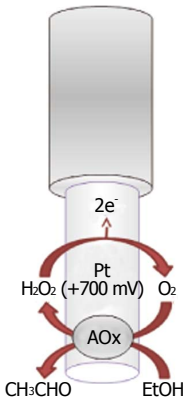

Figure 9 Schematic representation of the biosensor for the detection of exogenous ethanol in the brain of freely moving animals. EtOH: Ethanol; CH_3CHO: Acetaldehyde; AOx: Alcohol oxidase.

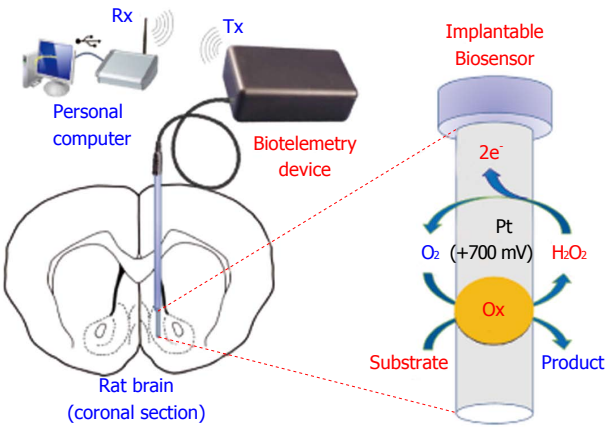

Figure 10 Schematic representation of the biotelemetry system, connected to a constant potential amperometry-based amperometric biosensor, for the real-time monitoring of brain neurochemistry in freely moving animals. Ox: Oxidase enzyme.

a period of two weeks. This neurochemical tool has been proven to be successful, especially when associated with a miniaturized telemetric system (see next paragraph). According to the results of previous studies[177,185,186], the ethanol biosensor has been demonstrated to be a reliable device for the short-time monitoring of exogenous ethanol in the CNS, and it could be used for studying ethanol pharmacokinetics during addiction and the real-time effect of drugs on ethanol levels in the CNS.

BIOTELEMETRY

Biotelemetry has be defined as the recording of physiological parameters by uni- or bidirectional electromagnetic signals[6,187], or more simply, it represents a variety of techniques intended for real-time monitoring of physiological parameters. Innovative biotelemetry systems (Figure 10) have been developed for studying brain neurochemistry[188], in particular for monitoring CNS dopamine in freely moving animals[189-191] and, more recently, in humans[192]. The wireless detection of dopamine requires complex waveform generation and high-resolution synchronization; indeed, as previously shown, FSCV allows the redox detection of dopamine up to ten times per second[189-191]. Also chronoamperometry and differential pulse voltammetry techniques have been demonstrated to work in conjunction with telemetric devices[158,193-196]; the resulting systems are very complex, not easily miniaturizable, and difficult to use in small rodents. On the contrary, non-pulsed techniques, such as CPA, free the microcontroller unit (MCU) from high-density calculations, allowing an increase in the number of implantable sensors and facilitating the miniaturization of the electronics[109,197]. The battery-powered biotelemetric device (Figure 10), composed of an amperometric module, an MCU, and a transmitter, polarizes the sensors and sends sensor data to a receiving unit connected to a PC. The system electronics exhibits low power consumption, high stability, and good linear response[3]. A CPA-based biotelemetry device may be easily interfaced with amperometric microsensors and biosensors[6,109,197] and leave enough MCU computing power available for other tasks such as motion detection using inertial physical sensors. Indeed, in a previous study, we described this new approach with the simultaneous detection of brain glucose, lactate, and movements in real time using a biotelemetric device fixed to the head of a freely moving rat[6].

COMPARISON BETWEEN VOLTAMMETRY AND MICRODIALYSIS

Although voltammetric techniques have been widely used in last decades, microdialysis still remains the "gold standard" for *in vivo* neurochemical study of the brain extracellular compartment. The advantages in using this technique include the possibility of measuring several neurochemicals at the same time with high sensitivity and very high selectivity, providing a more complete picture of the ECF. Its invasiveness, associated with low temporal resolution, and the necessity of using connecting tubes to carry out the experiments do not make it particularly suitable for monitoring fast neurochemical changes and do not allow the application of wireless techniques. As an alternative, electrochemical sensors are increasingly-used tools to study the neurochemical modifications in the ECF. The main characteristics of these devices are represented by very low invasiveness (carbon fibers in particular), when compared with microdialysis probes, and, most of all, their capability of monitoring variations of analytes in seconds or fractions. Furthermore, some electrochemical sensors have been demonstrated to be effective for weeks or months when implanted in the brain and, as described in this review, they are the optimal candidates for wireless detection. The Table 1 summarizes the principal characteristics of the main techniques indicated in this review.

CONCLUSION

Implantable (Bio)sensors, integrated into miniaturized telemetry systems, represent a new generation of analytical tools for studying brain neurochemistry of awake, freely

Table 1 Principal characteristics of the main techniques indicated in this review and used for *in vivo* monitoring of brain neurochemistry

Characteristics of the technique	Technique				
	Electrochemical techniques (voltammetry)				Microdialysis
	CPA	CA	DPV	FSCV	
Brain invasiveness	+	+	+	+	++
Selectivity	+	+	++	++	+++
Sensitivity	++	++	+	+	+++
Concentration range	nmol/L-mmol/L	nmol/L-mmol/L	nmol/L-mmol/L	nmol/L-mmol/L	fmol/L-mmol/L
Temporal resolution	++++	+++	++	+++	+
Spatial resolution	++	+++	++	+++	+
Monitoring period	d/wk	d/wk	d/wk	d/wk	h/d
Untethered detection	++	+	+	+	-

CPA: Constant potential amperometry; CA: Chronoamperometry; DPV: Differential pulse voltammetry; FSCV: Fast-scan cyclic voltammetry.

moving animals in real time. This approach, based on simple and inexpensive components, could be used as a rapid and reliable model for studying the physiology, the pathophysiology, and the effects of different drugs (or toxic compounds such as ethanol) on brain neurochemical systems.

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