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Simultaneous *In Vivo* Measurement of Dopamine, Serotonin and Ascorbate in the Striatum of Experimental Rats Using Voltammetric Microprobe

Samo B. Hocevar [1], Marko Zivin [2], Aleksandra Milutinovic [3], Marko Hawlina [4], Emily A. Hutton [1], and Bozidar Ogorevc [1]

[1] Analytical Chemistry Laboratory, National Institute of Chemistry, Hajdrihova 19, SI-1001, Ljubljana, Slovenia, [2] Institute of Pathophysiology, Faculty of Medicine, University of Ljubljana, Zaloška 4, SI-1000, Ljubljana, Slovenia, [3] Institute of Histology and Embryology, Faculty of Medicine, University of Ljubljana, Korytkova 2, SI-1000, Ljubljana, Slovenia, [4] Eye Clinic, University Medical Center, Zaloška 29, SI-1000, Ljubljana, Slovenia

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1. ABSTRACT

The *in vivo* performance of a voltammetric microprobe based on overoxidized poly(1,2-phenylenediamine) coated carbon fiber microelectrode (OPPD/CFME), developed in our laboratory, is presented. For this purpose, an OPPD microprobe was stereotaxicaly implanted in the striatum of a deeply anesthetized Wistar rat for the simultaneous measurement of dopamine, serotonin and ascorbate. Furthermore, the *post mortem* levels of these physiologically important compounds were monitored after the rats were terminated with an overdose of anesthetic introduced through an indwelling jugular catheter. Using cyclic (CV) and square-wave (SWV) voltammetry, the OPPD/CFME was demonstrated to exhibit efficient separation of the voltammetric signals of dopamine, serotonin and ascorbate in the presence of biological matrix, with the SWV mode allowing more convenient detection regarding both sensitivity and selectivity. Explicit proof of *in vivo* dopamine detection at the OPPD/CFME was achieved via the absence of the dopamine signal in rats with unilateral lesions of nigrostriatal dopaminergic neurons, which was induced by the use of the selective dopaminergic neurotoxin 6-hydroxydopamine (6-OHDA). In stark contrast to non-treated rats, where a strong signal corresponding to 1.2 μM dopamine was measured at ca. 90 s after the rats' death, the signal for dopamine in dopamine-depleted striatum of 6-OHDA rats was absent. A critical comparison of the *in vivo* performance of the OPPD/CFME to that of bare and Nafion-coated carbon fiber microelectrodes, often used in neurophysiological studies, clearly showed a significant advantage of the former microprobe. The OPPD/CFME allowed multiple and repetitive *in vivo* measurements, along with pre- and post-measurement external calibration, with no loss in selectivity and an acceptable loss in sensitivity, indicating that the active sensing sites were not adversely blocked by the components of the extracellular fluid, thus affirming the great practical *in vivo* applicability of the OPPD microprobe.

2. INTRODUCTION

Since the first pioneering attempts to perform *in vivo* probing of brain neurotransmitters (1,2), there has been growing interest in the development of new methods capable of providing unequivocal results on their basal and evoked levels. Among different approaches, microdialysis, liquid chromatography, fluorescence, and, in particular, electrochemical detection have been proven to be the most convenient techniques, since they can be successfully adapted to such a challenging environment and auspiciously coupled with required tool miniaturization (3,4). Considerable efforts have been focused upon the preparation of different micro- and ultramicro-(bio)sensors that can be potentially employed for reliable *in vivo* probing, such as single cell measurements (5) and in particular brain probing, where the highly complex extracellular environment represents a considerable challenge for the selective detection of neurotransmitters (6). When the species of interest are electrochemically active, e.g. several neurotransmitters and other physiologically important biomolecules, advanced electrochemical techniques, in conjunction with micro- and ultramicro-sensors, impart possibilities for direct, spot measurements, and successfully address issues concerning sensitivity, selectivity, time resolution, stability, and in particular the sensor's overall dimensions (7-10).

The first direct electrochemical *in vivo* measurements of neurotransmitters (dopamine) were based on an unmodified graphite paste electrode, where both the selectivity and sensitivity represented crucial limitations (11). Following these ambitious early studies, many electrochemical sensors, which coupled the unique properties of microelectrodes with different modification routes and/or pretreatment strategies in order to enhance the sensors' overall performance were proposed, and among which the introduction of the carbon fiber microelectrode represented a significant contribution to *in vivo* studies (12). When detecting selected neurotransmitters, such as the catecholamines dopamine, epinephrine and norepinephrine, and serotonin, histamine etc., that are positively charged at physiological pH, their detection can be seriously obscured by several extracellular interferents with similar oxidation potentials. Among them, ascorbate, uric acid, anionic biogenic metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid and 5-hydroxyindoleacetic acid (5-HIAA) are well-known as ubiquitous interferents when probing the aforementioned neurotransmitters (2,3). To enhance temporal resolution and to overcome undesirable effects arising from some of the interferents, the application of fast cyclic voltammetry in connection with a carbon fiber microelectrode was proposed (13,14). To improve the sensitivity and selectivity, several authors have suggested the electrochemical pre-anodization of the electrode surface (15,16), laser activation (17), the application of semi-permeable membranes and other modifiers (18-21), the introduction of new carbon electrode materials such as graphite reinforced with carbon (22) and very recently, carbon nanotubes (23-28). Negatively charged Nafion as a semi-permeable membrane has also been used for this purpose to suppress signals originating from negatively charged and some neutral chemical interferents, and to enhance the sensitivity (29-32). Despite all of these efforts, challenges still remain, in particular concerning direct, simultaneous *in vivo* measurements of several (bio)molecules of interest, and problems associated with the sensitivity, stability, etc. (21,22,24).

When probing neurotransmitters *in vivo*, it is of great importance to maintain/induce their release under certain control using different protocols. In general, their release can be induced electrically or chemically (9,31,32,33), while in conscious freely-moving animals fluctuations above basal levels may be related to the ongoing behavior. There are also some reports describing a massive release of dopamine and other biogenic amines in ischemic states, such as stroke or anoxia (34,35) and could thus also be detected immediately after death (36,37). The *post mortem* increase in the concentration level of dopamine in the brain has been attributed to its massive release due to the low energy state, diminished uptake in presynaptic terminals and reduced degradation due to reduced monoamine-oxidase activity (37). Dopamine, but not serotonin overflow induced in terminal regions, such as the striatum, by ischemic conditions (38) or dopamine overflow after intoxication by amphetamines (2,39) may significantly contribute to neurodegenerative changes. Furthermore, the extracellular fluid of the striatum contains a high level of ascorbate, an antioxidant vitamin known to have neuroprotective properties in experimental models of excitotoxic neurological disorders, including stroke, Huntington's disease, convulsive behavior and reactive species-related damage (40,41).

Recently, we proposed the preparation of the overoxidized poly(1,2-phenylenediamine) coated carbon fiber microelectrode (OPPD/CFME) for simultaneous measurement of dopamine and ascorbate at their physiological levels, under *in vitro* conditions (42). This voltammetric microprobe exhibited favorable performance characteristics with regard to selectivity and sensitivity towards dopamine and ascorbate. In the present study, we have expanded its scope and introduced the application of the OPPD/CFME to direct *in vivo* measurement of dopamine, serotonin and ascorbate in the striatum of deeply anesthetized rats, before and after the death of the experimental animals. The aim of the experiments was to evaluate the *in vivo* performance of the microprobe, including calibration, to make a critical comparison with the *in vivo* performance of bare and Nafion-coated carbon fiber microelectrodes that are often used in neurophysiological studies, and to examine the suitability of its reuse under *in vivo* and *post mortem* conditions.

3. MATERIALS AND METHODS

3.1. Apparatus

Cyclic (CV) and square-wave voltammetry (SWV) measurements were performed using an electrochemical workstation (Autolab PGSTAT12, Eco Chemie, Utrecht, The Netherlands) driven by GPES 4.9

software (Eco Chemie). For *in vivo* and *in vitro* experiments, a three-electrode configuration was employed consisting of either an overoxidized poly(1,2-phenylenediamine) (OPPD)- or a Nafion-coated or bare carbon fiber microelectrode (CFME) as the working electrode, with an AgCl coated silver wire pseudo-reference electrode for *in vivo* measurements or an Ag/AgCl/(KCl satd.) reference electrode in the case of *in vitro* measurements, while a platinum wire served as the counter electrode. *In vitro* experiments were carried out in a voltammetric cell at room temperature (23 ± 2 °C).

3.2. Reagents and solutions

Dopamine, L-ascorbic acid, and serotonin were supplied by Sigma and their respective standard solutions were prepared daily in 0.05 M phosphate buffer solution (pH 7.4), which also served as the supporting electrolyte in all *in vitro* measurements. Phosphate buffer solution was prepared by mixing appropriate amounts of di-potassium hydrogen phosphate and potassium di-hydrogen phosphate, both provided by Sigma, to obtain the desired pH value. All chemicals employed in this work were of analytical grade and used as received. Water used to prepare all solutions throughout the work was purified via an Elix10/Milli-Q Gradient unit (Millipore, Bedford, USA).

3.3. Microelectrode fabrication and modification

The fabrication of the substrate cylinder carbon fiber microelectrodes was accomplished by direct thermal sealing of a single carbon fiber (diameter 7 μm, Goodfellow Co., Oxford, UK) into a glass capillary tube employing a microelectrode puller (PP-830, Narishige, Tokyo, Japan) with final carbon fiber tip length of ca 0.5 mm. Some other details are given elsewhere (43). Optical inspections of the fabricated microelectrodes were accomplished using an inverted microscope (Eclipse, Nikon, Tokyo, Japan) equipped with a digital camera (Pixera, Los Gatos, CA).

An OPPD/CFME was prepared by electrochemical modification of a carbon fiber microelectrode in 0.05 M phosphate buffer solution (pH 7.0) containing 5 mM 1,2-phenylenediamine monomer (Sigma) and 0.1 M sodium dodecyl sulfate (Sigma) at +0.8 V for 20 min, followed by electrochemical overoxidation in a 0.05 M phosphate buffer solution (pH 7.0) by cycling the potential between 0.0 and +2.2 V at a scan rate of 10 V/s, for 50 cycles. Details regarding the OPPD microprobe preparation are given in our previous report (42). A Nafion-coated carbon fiber microelectrode was prepared by carrying out three consecutive dipping/drying cycles of a bare CFME in the Nafion solution, as described elsewhere (31). The Ag/AgCl pseudo-reference electrode was prepared by anodizing a silver wire (diameter 0.5 mm) in a 0.1 M HCl solution for 1 min at +1.0 V.

3.4. Animals and anesthesia

For the *in vivo* experiments in this work female Wistar rats weighing between 150 and 200 g were used. They were maintained on a 12 h light-dark cycle (light on: 07.00 - 19.00) in a temperature-controlled colony room at 22 – 24 ^{o}C with free access to rodent pellets and tap water. They were handled according to the National Veterinary Institute Guide for the Care and Use of Laboratory Animals.

The animals were anesthetized with the i.p. injection of 2% xylazine hydrochloride (8 mg/kg; Rompun, Bayer, Leverkusen, Germany) and ketamine hydrochloride (60 mg/kg; Ketanest, Parke Davies, Vienna, Austria). For the *post mortem* measurements, the rats were terminated with 0.5 mL of anesthetic injected through the intrajugular catheter.

3.5. Unilateral 6-hydroxydopamine (6-OHDA) lesions of the nigrostriatal pathway

The animals were placed in a stereotaxic frame (TrentWells, South Gate, CA) and 6-hydroxydopamine hydrobromide (8 μg of free base dissolved in 0.9 % saline containing 0.02 % ascorbic acid; RBI, Natick, MA) was infused at a rate of 1 μL/min over 4 min into the right medial forebrain bundle (MFB) at the following coordinates: 3 mm anterior from lambda, 1.2 mm lateral from the midline and 7.3 mm ventral from the surface of the dura (44). At each injection site the cannula was left in place for 2 min before retraction.

To assess the development of nigrostriatal degeneration, the apomorphine test was performed by treating the 6-OHDA lesioned animals with a directly acting mixed agonist of dopamine receptors apomorphine (0.5 mg/kg, *s.c.*) in the sixth post-operative week. Turning response was recorded by placing the rats in plastic cylindrical chambers (40 cm diameter) of the Lablinc automated rotometer system (Colbourn Instruments, Allentown, PA). Only the 6-OHDA rats that responded with peak turning frequency of at least 7 contralateral turns per minute were used in subsequent experiments.

3.6. Surgery and stereotaxic implantation of the voltammetric microprobes

Chronic indwelling jugular catheters (i.d. 0.5 mm, o.d. 1.0 mm) composed of a Silastic internal part (30 mm; Dow Corning Corporation, Midland, MI) and a polyurethane external part (90 mm; Micro-Renathane; Braintree Scientific, Braintree, MA) were implanted 5 days before stereotaxic implantation of the microprobes. The rats were deeply anesthetized, the right external jugular vein was isolated and the Silastic part of the catheter inserted through the vein into the right atrium. It was fixed to the jugular vein with a single stitch, and with another stitch to the underlying neck muscle. The external part of the catheter was passed subcutaneously to exit at the back of the neck area. At the exit the catheter was stoppered with an adapted needle (Microlance 3, 22G2, BD Medical Systems, Drogheda, Ireland). To maintain patency, 0.2 mL sterile saline containing heparin 500 IU/100 mL and gentamycin 12 mg/100 mL were injected daily through the catheter.

For the stereotaxic placement of the OPPD microprobes, the rats were deeply anesthetized and immobilized in a stereotaxic frame. The skull was then surgically exposed and a small diameter trepanation hole

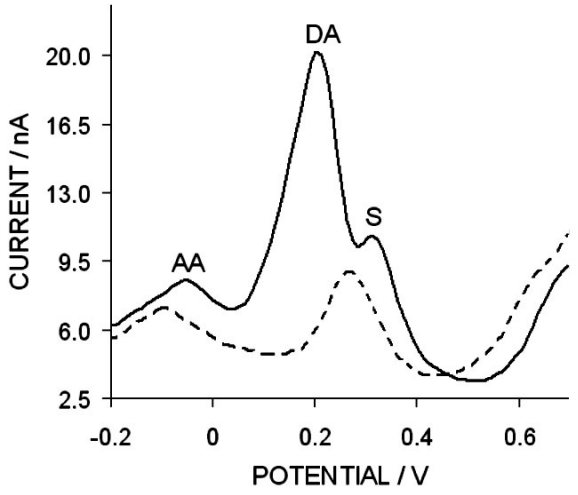

Figure 1. Square-wave voltammograms obtained at OPPD microprobe in the striatum of an anesthetized rat before (dashed line) and approx. 90 s after (solid line) death. SWV settings, potential scan from -0.2 to +0.7 V with a frequency of 10 Hz, potential step of 10 mV and pulse amplitude of 50 mV.

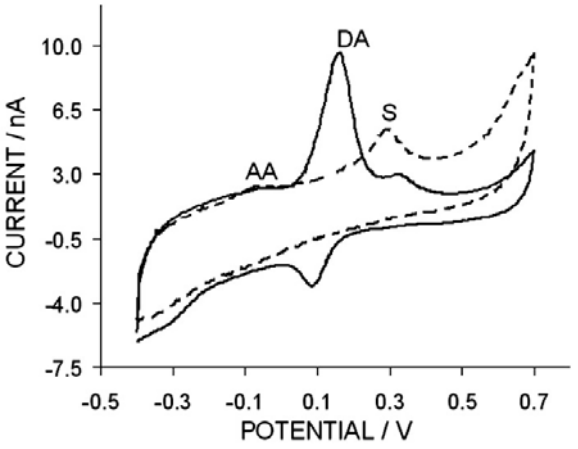

Figure 2. Cyclic voltammograms obtained at OPPD microprobe in the striatum of an anesthetized rat before (dashed line) and approx. 90 s after (solid line) death. CV settings, cyclic potential scan from -0.4 to +0.7 V at a scan rate of 100 mV/s.

was drilled into the skull for microprobe implantation. The OPPD/CFME was inserted, using a micromanipulator, in the caudate putamen at the following coordinates: 0.5 mm anterior from bregma, 3.0 mm lateral from the midline and 4.8 mm ventral from the surface of the dura. The Ag/AgCl pseudo reference and the platinum counter electrodes were placed under the skin of the scalp.

3.7. Measurement procedures

In vivo measurements were performed using either CV or SWV modes with the potential usually scanned from -0.4 to +0.7 V. The SWV operational setting was: frequency of 10 Hz, potential step of 10 mV and pulse amplitude of 50 mV, while in the CV mode a scan rate of 100 mV/s was applied. No microprobe cleaning was required between the successive *in vivo* runs. *In vitro* measurements were carried out in a quiescent 0.05 M phosphate buffer solution (pH 7.4) in the presence of dissolved oxygen with the same operational settings as those used for *in vivo* measurements.

4. RESULTS AND DISCUSSION

To examine the suitability of the proposed OPPD/CFME for direct voltammetric *in vivo* probing we first performed measurement of, as an example, released dopamine after the rats were terminated with an overdose of anesthetic, along with the concomitant monitoring of serotonin and ascorbate. Figure 1 shows square-wave voltammograms obtained in the striatum of an anaesthetized rat before (dashed line) and after (solid line) the death. The substantial release of dopamine, recorded approximately 90 s *post mortem,* can be clearly seen at ca. 0.20 V, whereas the level of ascorbate (at ca. -0.06 V) remained practically unchanged, while the voltammetric response to the basal level of serotonin (at ca. 0.30 V) slightly decreased after death with the signal partially overlapping with that corresponding to dopamine. Evidently, neither electrochemically active nor passive components of the present biological matrix interfered with the direct *in vivo* voltammetric imaging using the OPPD microprobe under the applied conditions. The *post mortem* increase of dopamine level has previously been observed, but has not been studied thoroughly using direct electrochemical probing (36,37). The observed massive release of dopamine was attributed to the coincident impaired uptake of dopamine and reduced monoamine-oxidase activity (37). In order to gain additional insight into the OPPD/CFME characteristics under *in vivo* conditions cyclic voltammetry was also used yielding, as expected (42), lower current responses to the species in focus but, on the other hand, giving raw current-voltage recordings. Cyclic voltammetry recordings presented in Figure 2 again revealed an increased *post mortem* dopamine concentration level (solid line) and exposed the known redox behavior of dopamine with a mid-potential at ca. 0.13 V, which shows a high degree of reversibility also under *in vivo* conditions. Cyclic voltammetry recordings clearly confirmed the practical insensitivity of the OPPD microprobe with respect to the extracellular fluid matrix.

To unequivocally verify the *in vivo* measurement of dopamine employing the OPPD microprobe, we conducted specific experiments using rats with unilateral lesions with 6-OHDA, which are devoid of dopamine on the lesioned side of brain. Figure 3A shows *post mortem* cyclic voltammograms recorded with the same OPPD/CFME consecutively in both the intact (solid line) and lesioned (dashed line) striatum. While the voltammogram obtained in the intact striatum exhibited a current signal originating from a substantial increase of the dopamine level, the voltammogram recorded in the dopamine depleted striatum did not exhibit any signal corresponding to dopamine. In addition, after the recordings displayed in figure 3A were completed, the

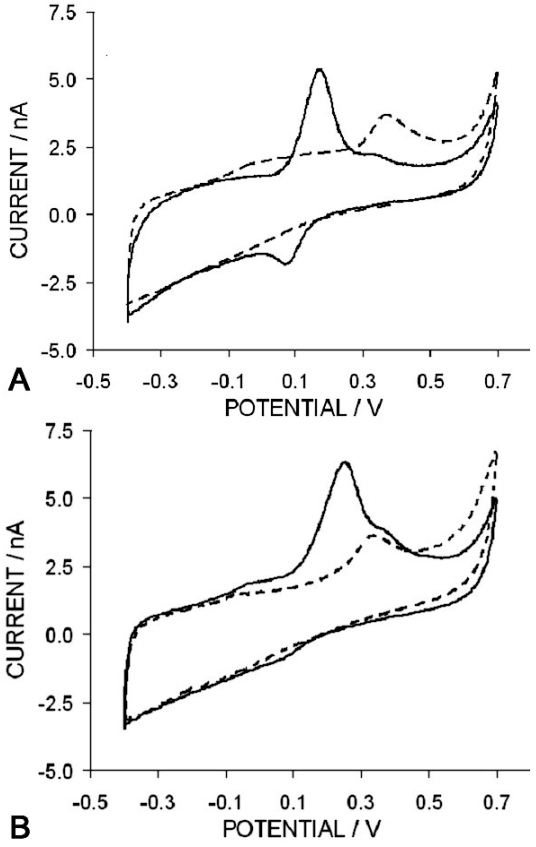

Figure 3. Cyclic voltammograms all recorded at the same OPPD microprobe (A) *post mortem* in the intact (solid line) and lesioned (dashed line) striatum, (B) *post mortem* after the microprobe was positioned back into the intact striatum (solid line), and (B) *pre mortem* in the intact region (dashed line) of the same anesthetized rat. Other conditions as in figure 2.

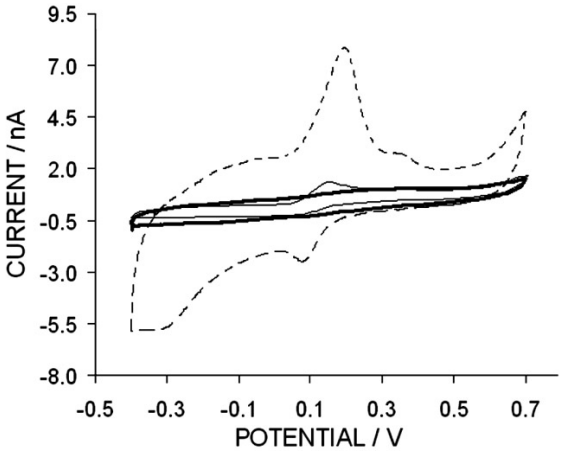

Figure 4. Cyclic voltammograms obtained *post mortem* in rat striatum at a bare carbon fiber microelectrode (thick solid line), at a Nafion-coated carbon fiber microelectrode (solid line), and at an OPPD/CFME (dashed line). Other conditions, as in figure 2.

OPPD microprobe was immediately transferred back into the intact striatum, where the signal for dopamine was again clearly recorded, as can be seen in figure 3B (solid line). For comparison, in figure 3B a *pre mortem* cyclic voltammogram (dashed line) recorded in the intact region employing the same microprobe, is also shown. A slight positive potential shift of the signal corresponding to dopamine in comparison to that in figure 3A is attributed to the *post mortem* change of pH (37). These results not only incontrovertibly proved the *in vivo* measurement of dopamine using the OPPD microprobe but also demonstrated the capability of repetitive usage of the OPPD microprobe under *in vivo* conditions, with no apparent memory effect.

An important aim of this work was to critically compare the OPPD microprobe with the *in vivo* voltammetric performance of bare and Nafion-coated carbon fiber microelectrodes, which are often used in neurophysiological studies, under the same conditions. The results of this comparison are displayed in Figure 4. As can be clearly seen, the bare carbon fiber microelectrode (thick solid line) exhibited a relatively low and poor voltammetric response accompanied with a high background contribution along with a lack of selectivity. The current signal appearing at potentials around 0.2 V is broad, evidently encompassing the current contributions originating from all three analytes and possibly some other potentially present electrochemically active compounds (e.g. metabolites). The response obtained at the Nafion-coated carbon fiber microelectrode (solid line) is higher in comparison to that obtained at the bare carbon fiber microelectrode, but still significantly lower when compared to that obtained at the OPPD/CFME (dashed line). The current signal obtained at the Nafion-coated microelectrode is attributed mainly to dopamine. This is supported by a reversible-like behavior and the fact that the Nafion membrane is negatively charged and thus rejects anionic species, such as ascorbate, and at the same time has the ability to preconcentrate cationic species, such as dopamine (23,32). In contrast, the OPPD microprobe exhibits three separate signals (dashed line) corresponding to ascorbate, dopamine and serotonin, as described above, together with significantly enhanced sensitivity. This comparison of the three voltammetric microprobes that vary in surface design clearly demonstrated the *in vivo* operational advantage of the proposed OPPD/CFME, in terms of both important sensing parameters - selectivity and sensitivity. Moreover, these results also illustrate that *in vivo* measurements of dopamine using unpretreated bare carbon fiber microelectrodes might be questionable when performed under the same or similar conditions as applied in this work.

The calibration of a microprobe is considered to be a critical point in evaluating the concentration levels of an analyte in focus, measured under *in vivo* conditions. Therefore, we also preliminarily examined the possibility of calibrating the OPPD/CFME, in order to estimate the levels of e.g. *post mortem* dopamine. We first performed *in vitro* pre-calibration of the OPPD microprobe, then

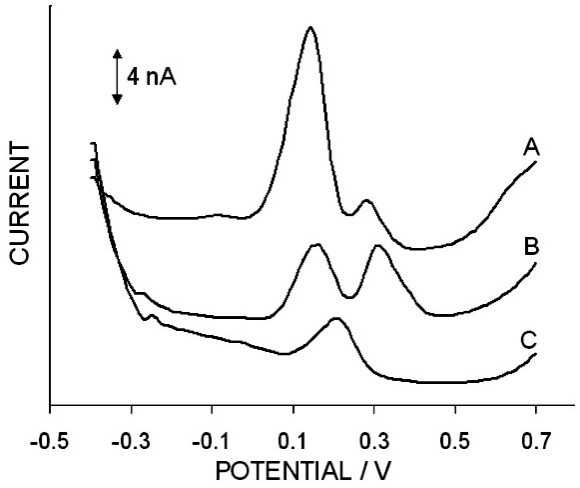

Figure 5. Square-wave voltammograms obtained at the same OPPD/CFME in (A) *post mortem* striatum of an overanesthetized rat, (B) pre-calibration solution for 300 nM dopamine and 250 nM serotonin, and (C) post-calibration solution, after the *in vivo* measurement, for 300 nM dopamine. Pre- and post-calibration solution: 0.05 M phosphate buffer (pH 7.4); potential scan: from -0.4 to +0.7 V. Other conditions as in figure 1.

conducted *in vivo* measurements, and finally performed *in vitro* post-calibration, all with the same OPPD microprobe. The voltammograms of this three-step procedure, presented in figure 5, were obtained in the striatum of an overanesthetized rat (A), in pH 7.4 phosphate buffer solution with 300 nM dopamine and an addition of 250 nM serotonin (B), and again for *in vitro* post-calibration with 300 nM dopamine (C). Serotonin was added (B) to illustrate the selectivity feature of the OPPD/CFME for dopamine and serotonin before its first *in vivo* application. On the basis of this calibration protocol, the *post mortem* dopamine level was quantified to be approximately 1.2 μM, which is consistent with earlier observations and predictions (36,37,45). We also estimated that the basal dopamine level in the striatum of deeply anesthetized rats, from SWV measurements as e.g. shown in figure 1, was below ca. 25 nM, i.e. below the limit of detection of the OPPD microprobe, and such a low level might be a consequence of the effect of the applied anesthetic. As can be seen from figure 5 (C), the post-calibration voltammogram revealed that the shape of the dopamine signal after the *in vivo* measurements, in comparison to that from the pre-calibration voltammogram (B), remained practically unchanged with only a slight decrease in the signal height. This may be ascribed to a limited fouling activity of some present matrix components such as lipids and/or proteins, but it apparently did not affect the overall performance of the OPPD microprobe. Although certain sophisticated but potentially important parameters of *"in vivo"* voltammetric calibration, such as temperature difference compensation and *in vitro* biological matrix simulation, were not taken into account in this preliminary calibration protocol examination, these experiments clearly illustrated the advantageous characteristics of the OPPD microprobe in its capability to perform multiple and repetitive *in vivo* measurements in combination with the pre- and post-calibration protocol. This is extremely important for *in vivo* measurements, where the standard addition method is not possible.

5. CONCLUSIONS

In this work the favorable performance of the OPPD microprobe for direct, simultaneous, *in vivo* measurement of selected neurotransmitters dopamine and serotonin, together with ascorbate, is presented. Using cyclic and square-wave voltammetry detection modes, the apparent insensitivity of the OPPD/CFME to real biological matrix was demonstrated with *in vivo* measurements of these compounds in the brain of experimental rats and the *post mortem* measurements revealed a massive release of striatal dopamine while serotonin and ascorbate concentrations were practically unchanged. In a critical comparison, the superior *in vivo* performance of the OPPD microprobe over bare and Nafion-coated carbon fiber microelectrodes was shown, allowing its multiple/repetitive *in vivo* application without significant loss in sensitivity and selectivity. In addition, a preliminary pre- and post-calibration protocol was established, which revealed the minor effects of the extracellular matrix upon the microprobe overall performance. Further *in vivo* use of the OPPD microprobe in freely movable rats and in some neurophysiological studies is considered and in progress.

6. ACKNOWLEDGMENTS

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Send correspondence to: Dr. Bozidar Ogorevc, Analytical Chemistry Laboratory, National Institute of Chemistry, Hajdrihova 19, SI-1001, Ljubljana, Slovenia, Tel: 386 1 4760 230, Fax: 386 1 4760 300, E-mail: bogorevc@ki.si

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