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Article

Time-Dependent Monitoring of Dopamine in the Brain of Live Embryonic Zebrafish Using Electrochemically Pretreated Carbon Fiber Microelectrodes

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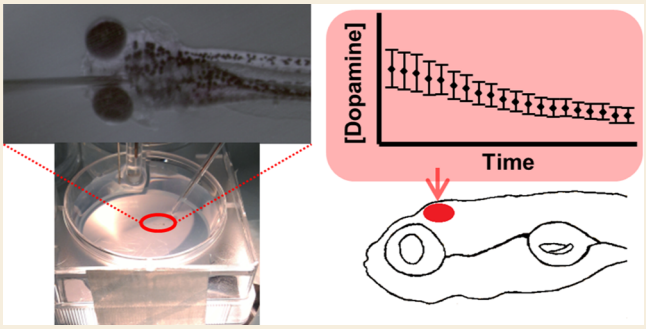
ABSTRACT: Neurotransmitters are involved in functions related to signaling, stress response, and pathological disorder development, and thus, their real-time monitoring at the site of production is important for observing the changes related to these disorders. Here, we demonstrate the first time-dependent quantification of dopamine in the brains of live zebrafish embryos using electrochemically pretreated carbon fiber microelectrodes (CFMEs) utilizing differential pulse voltammetry as the measurement technique. The pretreatment of the CFMEs in 0.1 M NaOH held at a potential of +1.0 V for 600 s improves the sensitivity toward dopamine and allows for reliable measurements in low ionic strength media. We demonstrate the measurement of extracellular dopamine concentrations in the zebrafish brain during late embryogenesis. The extracellular dopamine concentration in the tectum of zebrafish varies between 200 and 400 nM. The conventional pharmacological manipulation of neurotransmitter levels in the brain demonstrates the selective detection of dopamine at the implantation site. Exposure to the dopamine transporter inhibitor nomifensine induces an increase in extracellular dopamine from 201.9 (±34.9) nM to 352.2 (±20.0) nM, while exposure to the norepinephrine transporter inhibitor desipramine does not lead to a significant modulation of the measured signal. Furthermore, we report the quantitative assessment of the catecholamine stress response of embryos to tricaine, an anesthetic frequently used in zebrafish assays. Exposure to tricaine induces a short-lived increase in brain dopamine from 198.6 (±15.7) nM to a maximum of 278.8 (±14.0) nM. Thus, *in vivo* electrochemistry can detect real-time changes in zebrafish neurochemical physiology resulting from drug exposure.



KEYWORDS: *carbon fiber microelectrode, neurotransmitters, dopamine, tricaine, zebrafish embryo, differential pulse voltammetry*

INTRODUCTION

The direct detection of biomarkers at their site of production requires the development of miniaturized probes that can reach and perform measurements at specific locations in live biological models. Electrochemical techniques have been successfully used to detect, identify, and quantify the concentration changes of analytes associated with physiological and pathological pathways in live organisms.[1−3] Such measurements are of particular interest in studying neurotransmitter dynamics and their involvement in stress response, cognition, memory, and pathological disorders.[4,5] Although the use of *in vivo* electrochemistry to measure changes in neurotransmitter concentration has been established, the sensitivity and selectivity of microelectrodes to track the release at the individual organ system level in different models and disease states is still challenging.[3,6−8]

The use of the zebrafish model system has increased in recent years to include the screening of environmental contaminants, drug development, and disease modeling.[9−11] Some of the advantages of using this model are low maintenance cost, high fecundity, rapid *ex utero* embryonic development, ease of manipulation, and visual access to major organs due to optical transparency.[12] Moreover, zebrafish offer a high degree of homology with the human genome and physiological and anatomical similarities with advanced organisms, including humans.[13] A functional nervous and digestive system develops in less than one week post-fertilization, allowing easy accessibility to implantation sites.[14] However, because of their extremely small size, the real-time detection of neurotransmitters in the organs of zebrafish embryos requires the fabrication of small-sized, highly specific sensors with good temporal and spatial resolution. For the past

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few years, our group has studied the neurotransmitter dynamics in the intestine of zebrafish embryos using implanted carbon fiber microelectrodes (CFMEs). Analytes such as nitric oxide[15] and serotonin[16] have been measured with good spatial resolution, indicating differential concentrations and distribution along the intestine. The upregulation or downregulation of these analytes as a result of pharmacological manipulation or the exposure to environmental contaminants has been demonstrated.[17−20] Additionally, fast-scan cyclic voltammetry at CFMEs has been used for neurochemical *ex vivo* measurements in brain slices and the whole brain of adult zebrafish and in zebrafish eye retina.[21−24] Shang *et al.* have used constant potential amperometry to demonstrate time-dependent dopamine release in the brain of larval zebrafish as a result of olfactory stimulation.[25]

Here, we report the time-dependent quantification of dopamine in the brains of live zebrafish embryos using an electrochemically treated CFME and differential pulse voltammetry (DPV) technique. To improve the sensitivity toward dopamine, microelectrodes were treated before use by constant potential amperometry in 0.1 M NaOH. The electrochemical treatment improved the detection performance toward catecholamines, particularly for dopamine, and less for epinephrine and norepinephrine, in comparison with interfering species such as serotonin. Moreover, we have used repetitive scan DPV to obtain a better resolution and differentiate between different neurotransmitters based on their oxidation potentials while still maintaining good temporal resolution. Using this approach, the extracellular dopamine levels in the brains of zebrafish embryos have been measured. To our knowledge, this is the first study to use electrodes treated in NaOH for the *in vivo* measurement of dopamine using DPV. The origin of the electrochemical signal was validated by the pharmacological manipulation of the dopamine dynamics with nomifensine, a dopamine uptake inhibitor, and desipramine, a norepinephrine uptake inhibitor. Furthermore, we have assessed the variation of brain dopamine concentration at the same implantation site upon the exposure of embryos to tricaine, an anesthetic agent used in zebrafish assays.

These results demonstrate that electrochemically treated microelectrodes are a powerful tool to quantitatively assess the evolution of dopamine in the brains of zebrafish embryos using DPV. Such measurements can be used to study a variety of neurodevelopmental mechanisms, to provide a greater understanding of the role of dopamine signaling in zebrafish and potentially other vertebrates, and to detect neurochemical changes due to drug exposure.

METHODS

Materials and Reagents

Carbon fibers (∼5 μm in diameter) were obtained from World Precision Instruments. Silver conductive epoxy was purchased from MG Chemicals. Five-minute nonconductive epoxy was obtained from Devcon. L-ascorbic acid sodium salt, dopamine hydrochloride, norepinephrine hydrochloride, tricaine (ethyl 3-aminobenzoate methanesulfonate salt), desipramine hydrochloride, and agar were purchased from Sigma-Aldrich. Calcium chloride was obtained from Acros Organics. 2-Propanol, sodium hydroxide, sodium chloride, and magnesium sulfate were purchased from Fisher Scientific. Sodium phosphate dibasic was purchased from Spectrum. Potassium phosphate monobasic and potassium chloride were purchased from LabChem, Inc. Serotonin hydrochloride was purchased from Alfa Aesar. Epinephrine hydrochloride was obtained from MP Biomedicals. Dimethylsulfoxide (DMSO) was purchased from J. T. Baker. Nomifensine maleate salt was obtained from ChemCruz. 0.1 M phosphate buffer (PB), pH 7.5, was prepared by mixing sodium phosphate dibasic and potassium phosphate monobasic. E3 medium (pH 6.9−7.2) containing 5 mM sodium chloride, 0.17 mM potassium chloride, 0.33 mM magnesium sulfate, and 0.33 mM calcium chloride was prepared in deionized water. All the solutions were prepared using purified water (18 MΩ, Millipore, Direct-Q System).

Instrumentation

Electrochemical measurements were performed using a CH1030A electrochemical analyzer (CH Instruments, Inc.). All the experiments were carried out in a three-electrode electrochemical cell equipped with a Ag/AgCl/1 M KCl reference electrode (CH111, CH Instruments, Inc.) and a platinum wire counter electrode. The working electrode was a custom-made modified CFME fabricated from a single carbon fiber. An optical microscope (Nikon SMZ1000 Stereomicroscope) was employed during the preparation of microelectrodes and zebrafish embryo manipulation during *in vivo* experiments.

Preparation and Characterization of Conditioned CFMEs

CFMEs were prepared according to our previously reported procedure.[18] The fiber at the tip of the electrode was cut ∼100 μm short under the microscope. The microelectrodes were cleaned by dipping in isopropanol for 1 min and by repeated fast-scan cyclic voltammetry between −0.4 and 1.4 V, 500 V/s, in 0.1 M PB, until a stable voltammogram was obtained. An amperometric conditioning step in 0.1 M NaOH was employed in order to improve the electrochemical performance of the sensor toward catecholamines. The working potential and duration of the conditioning step were optimized using dopamine as a model analyte. The working potential was varied between +1.0 and +1.4 V, and the duration between 600 and 1800 s. After preparation, the conditioned CFMEs were treated by DPV in 0.1 M PB until a stable baseline was obtained, using the following parameters: 4 mV potential increment, 50 mV pulse amplitude, 50 ms pulse width, and 200 ms pulse period. The duration of one scan is 35 s. For measurements, a background scan was obtained before all the measurements in the absence of analyte, and it was subtracted from each voltammogram recorded in the presence of analyte. A cleaning step consisting of 1 s of amperometry at +1.4 V was employed in between measurements. The characterization of the CFME was done in 0.1 M PB for physiological concentrations of typical neurotransmitters, including dopamine, serotonin, epinephrine, norepinephrine, and ascorbate. A calibration curve for dopamine was recorded *in vitro* in E3 medium as the electrolyte solution was used for the *in vivo* study.

Surface Characterization

Atomic force microscopy (AFM) images were recorded using a Bruker MM8 instrument with NCHV-A tips (Bruker) using a scan rate of 1 Hz. The images were processed using Bruker's NanoScope Analysis software. Images were fitted with third-order plane fitting and the R_a parameter value was determined using the roughness tool. Raman spectra were collected using an inVia Raman microscope (Renishaw, UK) equipped with a 514 nm Ar laser for 10 accumulations and 10 s of exposure time using 100% laser power. The effective surface area of the CFMEs was determined in the presence of $K_4[Fe(CN_6)]$ according to the procedure reported by Lu *et al.*[26]

Fish Stock

Fish maintenance and matting were performed as previously described.[27] AB wild-type fish were used for all the procedures. Zebrafish eggs were collected immediately after fertilization and sorted out in E3 medium. At 24 h post-fertilization (hpf), developing embryos were manually dechorionated. Embryos were separated into 6-well plates, 10 embryos per well, and grown in E3 medium until 5 days post-fertilization (dpf), when they were used for *in vivo* measurements. All the animals were handled in strict accordance

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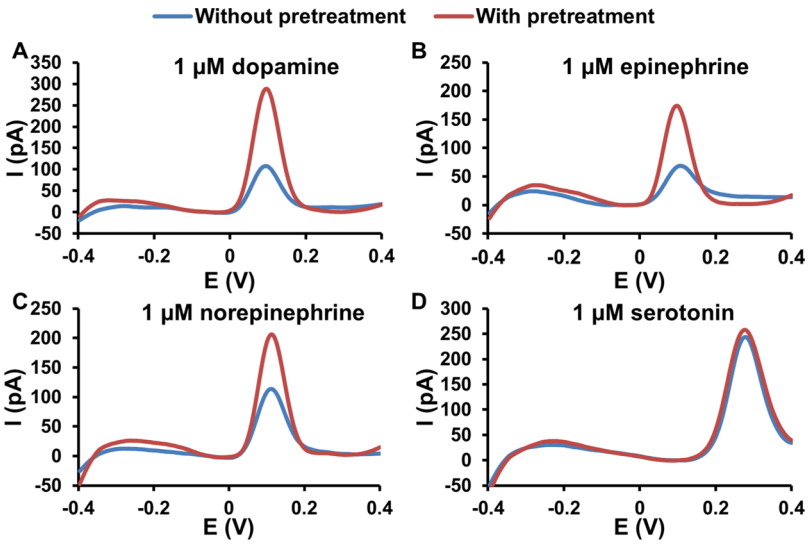

Figure 1. Comparison of the voltammetric response recorded before and after the pretreatment of CFMEs by constant potential amperometry at +1.0 V in 0.1 M NaOH for 600 s in the presence of 1 μM dopamine (A), epinephrine (B), norepinephrine (C), and serotonin (D). The DPV parameters were as follows: 4 mV potential increment, 50 mV pulse amplitude, 50 ms pulse width, and 200 ms pulse period. Only the first scan recorded is shown. All the voltammograms are background subtracted.

with good animal practice as defined by national (NIH Office of Laboratory Animal Welfare) and local (Clarkson University Institutional Animal Care and Use Committee) bodies, and all the work were approved by the appropriate committee.

In Vivo Measurement Protocol

Live 5 dpf zebrafish embryos were washed in E3 medium before use. Each embryo was immobilized onto an agarose gel plate under the microscope and covered with E3 medium. A background DPV scan was measured in E3 medium, followed by the insertion of the microelectrode using a micromanipulator into the tectum region of the mesencephalon[28,29] at a depth equal to the protruding length of the carbon fiber. One *in vivo* measurement sequence consisted of 20 repetitive DPV scans, each preceded by a 1 s cleaning step at 1.4 V and a 5 s quiet time. The total measurement time was 820 s. CFMEs were tested *in vitro* before and after each measurement sequence using 1 μM dopamine.

Pharmacological Manipulation

In validation experiments, nomifensine and desipramine were used to modulate the level of dopamine. During a measurement sequence, the nomifensine or desipramine stock solution in DMSO were added to the bath at 410 s. The final concentration of each drug in E3 medium was 320 μM.

Anesthesia Protocol

For anesthesia experiments, a fixed volume of the tricaine stock solution in deionized water was added during the measurement at 410 s in the near proximity of the embryo. The final concentration of tricaine in the bath was 0.5 mM. Embryos were assessed for active heart beating during the experiments.

Statistical Analysis

The results are expressed as the mean ± standard error of the mean (n = number of replicate experiments). Statistical significance was evaluated using the one-way analysis of variance (ANOVA) with a post-hoc Tukey HSD test or paired t-test. The asterisks denote statistically significant results. One (*) and two (**) asterisks indicate statistical significance at $p < 0.05$ and $p < 0.01$, respectively.

RESULTS AND DISCUSSION

Optimization and *In Vitro* Calibration of Electrochemically Pretreated Microelectrodes

Electrochemical conditioning of carbon electrodes has been previously used to change the nature and properties of the carbon surface and improve detection capabilities toward biological analytes.[30] The overoxidation of the carbon fiber during fast-scan cyclic voltammetry treatment has been shown to increase dopamine adsorption by the introduction of negatively charged functional groups capable of interacting with positively charged analytes.[31] However, this leads to continuous erosion of the carbon surface due to carbon dioxide evolution and the removal of particulate carbon.[32] Alternatively, overoxidation in basic solutions can permanently affect the properties of the carbon material in a controlled manner. Although electrochemical treatment in basic solutions at high potentials was employed for complete etching purposes,[33−35] the use of mild overoxidation without inducing structural damage to the carbon fiber structure was reported to help improve sensitivity and selectivity. Mild pretreatment in sodium hydroxide solutions was suggested to improve the redox properties of carbon electrodes toward nitric oxide without the use of a catalytic material.[36,37] The selective detection of ascorbate secretion from single cells was achieved by the pretreatment of CFMEs in a buffered electrolyte solution, pH 9.5.[38]

Figure 1 shows DPVs recorded in the presence of typical neurotransmitters at the CFME before and after pretreatment by constant potential amperometry at +1.0 V in 0.1 M NaOH. Higher oxidation currents without variation in the oxidation potential were observed at pretreated CFMEs, particularly for dopamine and for epinephrine and norepinephrine. However, the oxidation of serotonin has not changed at pretreated microelectrodes. The increase in oxidation currents observed for the first three analytes could be explained by an increase in the active surface of the electrodes due to the formation of defects and cracks in the structure of the carbon fiber.[36,39] AFM images of the NaOH-pretreated CFMEs show more

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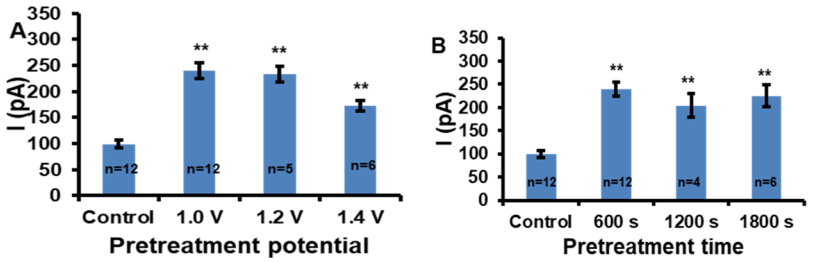

Figure 2. Optimization of alkaline pretreatment potential (A) and time (B). The signal showed is the average response to 1 μM dopamine measured by DPV (4 mV potential increment, 50 mV pulse amplitude, 50 ms pulse width, and 200 ms pulse period). The error bars represent the standard error of the mean for "*n*" independent microelectrodes. Statistical significance in comparison with the control experiment is calculated by the one-way ANOVA with the post-hoc Tukey HSD test and it is indicated by one (*) and two (**) asterisks at $p < 0.05$ and $p < 0.01$, respectively.

roughness in comparison with the smooth surface of CFMEs without pretreatment (Figure S1A–C). Raman spectra analysis indicates the CFMEs pretreated with NaOH exhibit more edge-plane carbon sites due to a decrease in the disorder level of the graphitic structure associated with increased I_D/I_G ratios (Figure S1D). This also suggests that there are more oxygen-containing functional groups at the carbon surface after the NaOH treatment, which is beneficial for increasing the adsorption of dopamine.[40,41] Additionally, the effective surface area determined in the presence of $K_4[Fe(CN_6)]$ is approximatively threefold higher at NaOH-pretreated CFMEs (Figure S1E). This indicates mild overoxidation in the presence of NaOH improves the electrochemical properties of the carbon surface for the oxidation of inner-sphere redox active analytes such as dopamine.[42] The fact that serotonin does not show improved sensitivity at NaOH-pretreated CFMEs may be due to its more complex oxidation pathways, which include side reactions forming secondary products that contribute to surface fouling.[43] Overall, the mild overoxidation of CFMEs in alkaline medium leads to a permanent change in surface properties and an improvement in sensitivity toward dopamine specifically.

The effect of pretreatment parameters such as potential and time was assessed. The pretreatment potential was varied between 1.0 and 1.4 V, while keeping the pretreatment time constant at 600 s. The use of a mild overoxidation potential (1.0 V) led to a 2–3 fold sensitivity increase in comparison with CFMEs without pretreatment (Figure 2A). With the increase in the pretreatment potential, the response to 1 μM dopamine decreased significantly, indicating a potential passivation of the surface. A pretreatment potential of 1.0 V was chosen as the optimal value. The pretreatment time was varied between 600 and 1800 s for a pretreatment potential of 1.0 V (Figure 2B). For all the time periods tested, the oxidation current for 1 μM dopamine increased significantly in comparison with the control CFMEs. However, increasing the pretreatment time over 600 s was not beneficial. Therefore, 600 s was chosen as the optimal pretreatment period.

Microelectrodes prepared by the optimized pretreatment procedure were tested in the presence of other neurotransmitters, including epinephrine, norepinephrine, serotonin, and ascorbate (Figure 3). Although for dopamine there is a significant increase in oxidation currents, a smaller increase in sensitivity is observed toward epinephrine. No statistically significant change is observed for norepinephrine and serotonin, while ascorbate cannot be detected at either microelectrode due to the restrictive potential window. Overall, the pretreatment of CFMEs significantly improves the sensitivity toward dopamine, with little change toward the

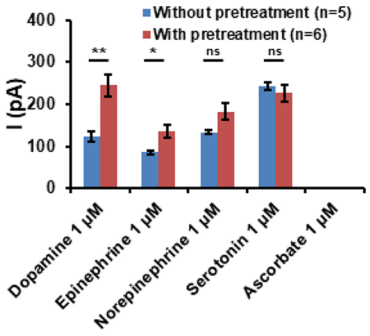

Figure 3. Comparison of the voltammetric response recorded before and after the alkaline pretreatment of CFMEs for 1 μM dopamine, epinephrine, norepinephrine, serotonin, and ascorbate. The signal shown is the average response measured for each analyte by DPV (4 mV potential increment, 50 mV pulse amplitude, 50 ms pulse width, and 200 ms pulse period). Ascorbate was not detected within the used potential window. The error bars represent the standard error of the mean for "*n*" replicate experiments. Statistical significance between the two groups is calculated using the one-way ANOVA with the post-hoc Tukey HSD test and it is indicated by one (*) and two (**) asterisks at $p < 0.05$ and $p < 0.01$, respectively, or by "ns" for no statistical significance.

other catecholamines and interfering compounds. Fouling of carbon surfaces is possible when detecting dopamine. This issue is typically solved by employing polymer-functionalized CFMEs.[44,45] However, we have observed the pretreatment procedure contributes to improving the antifouling properties of the carbon surface. The decay of the dopamine signal after repeated DPV recordings without any cleaning step is less pronounced at alkaline-pretreated CFMEs in comparison with normal microelectrodes (Figure S2). Additionally, for calibration and *in vivo* measurements purpose, a cleaning step between repetitive DPV scans was employed, consisting of 1 s of polarization at +1.4 V. The cleaning step was shown to improve the reproducibility of the signal for the repetitive measurement of 1 μM dopamine, with little signal decay from fouling (Figure S3). This cleaning step is independent of the initial pretreatment procedure performed in alkaline medium.

The pretreated CFMEs were tested in both PB and E3 medium and compared. In E3 medium, the dopamine oxidation peak is slightly shifted to higher potentials (Figure 4A). We attribute this effect to the fact that E3 medium has a lower pH and lower ionic strength in comparison with PB. The pH value of the medium has been suggested to influence the shifting of oxidation peaks in voltammetric data.[46,47] The significant difference between the ionic strength values of PB and E3 medium is, however, the main factor behind the peak

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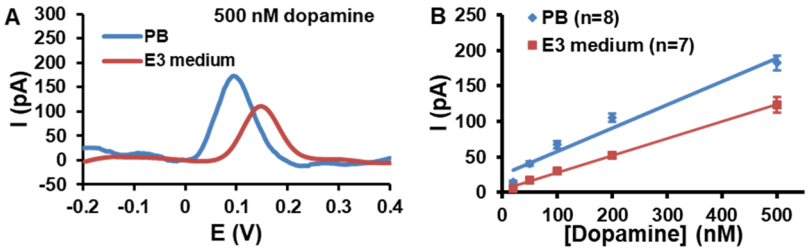

Figure 4. (A) Comparative response of pretreated CFMEs to 500 nM dopamine in PB and E3 medium. (B) Calibration curves for dopamine detection at pretreated CFMEs recorded in PB (R^2 = 0.963) and E3 medium (R^2 = 0.998). The signal showed is the average response measured for the varied concentrations of dopamine by DPV (4 mV potential increment, 50 mV pulse amplitude, 50 ms pulse width, and 200 ms pulse period). The error bars represent the standard error of the mean for "*n*" replicate experiments.

shift and broadening.[48−50] This observation further supports the hypothesis that the pretreatment procedure contributes to a complex change in the electrochemical properties of the carbon surface, affecting its performance in the media of different pH and ionic strength values. Furthermore, the pretreated CFMEs responded linearly to dopamine concentrations between 20 and 500 nM in both the PB and E3 medium (Figure 4B). However, the sensitivity toward dopamine in E3 medium drops significantly to 0.24 pA/nM, in comparison with 0.33 pA/nM in PB. As suggested, this is due to the lower pH and lower ionic strength of the E3 medium. The superior sensitivity of the pretreated CFMEs toward dopamine is therefore an advantage for performing *in vivo* measurements in conditions with restrictive electrolyte concentrations and low ionic strength.

Extracellular Dopamine Detection in Live Zebrafish Embryos and Pharmacological Validation

The development of the neuronal pathways in zebrafish starts at the earliest stages of embryogenesis.[51] The catecholaminergic system is distributed in discrete sections of the brain as clusters of cells.[52−54] As all the neuronal populations can be detected by 96 hpf,[55] zebrafish embryos are an advantageous animal model to study neurological pathways during embryogenesis.[56] Specifically for dopamine release, several groups of dopaminergic neurons develop across the mesencephalon, in the tectum.[28] Shang *et al.* have implanted an electrochemical sensor in the optic tectum of zebrafish larvae and have confirmed the presence of dopamine only.[25] Therefore, we have selected the same region as our implantation site (Figure 5A,B).

A typical voltammogram recorded in the tectum of 5 dpf embryos exhibits a clear oxidation peak at ∼ 0.2 V, consistent with the potential where dopamine is oxidized (Figure 5C). The shifted potential of dopamine toward higher values and the broadening of the peak in comparison with the *in vitro* measurement is driven by the complex chemical environment at the implantation site and the fact that the reference electrode is placed outside of the tissue. The average time-dependent concentration profile of dopamine in the tectum was determined based on the *in vitro* calibration curve (Figure 5D). The extracellular dopamine concentration stabilizes after 400 s to about 300−400 nM. The higher concentration of dopamine at the beginning of the measurement indicates the accumulation of extracellular dopamine at the measurement site due to the damage to neuronal tissues during implantation. For this reason, the signal was allowed to stabilize for 10 scans before any chemical stimulation was applied to the bath. The variation of the measured dopamine concentration from one embryo to another is the result of the inherent differences

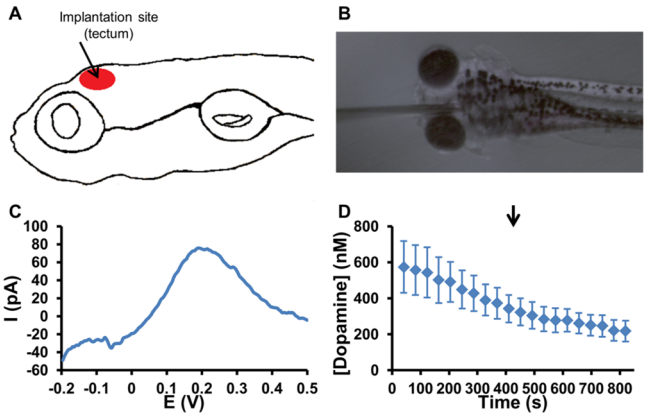

Figure 5. (A) Graphical representation of the implantation site in the tectum of 5 dpf zebrafish embryos. (B) Microscope image of a CFME implanted in the tectum of 5 dpf zebrafish embryos. (C) Typical DPV recorded at the implantation site in a 5 dpf zebrafish embryo exhibits an oxidation peak at 0.2 V associated with the oxidation of dopamine. (D) Average dopamine concentration−time trace measured in the tectum of 5 dpf zebrafish embryos. The arrow indicates the injection of deionized water in the near proximity of the embryos (control experiment) at 410 s. The error bars represent the standard error of the mean for n = 6 replicate experiments in individual embryos.

between individual embryos, the slight variability of the implantation site and depth and the variable performance of the microelectrodes. The injection of a control sample (deionized water) in the near proximity of the embryo did not lead to any significant change in the concentration of dopamine. This shows that the neurochemical activity in the tectum is not perturbed by physically or chemically inert stimuli. Furthermore, the performance of the microelectrodes is well-maintained after each use, as indicated by the comparison between pre- and post-calibrations performed *in vitro* (Figure S4).

In order to pharmacologically confirm the identity of the electrochemical signal, embryos were treated with nomifensine during measurements. Nomifensine is a well-established dopamine uptake inhibitor that acts by blocking the dopamine transporters, thus increasing the synaptic dopamine concentration measured at the implanted microelectrode.[57] An excess concentration of nomifensine was injected in the near proximity of the embryo in order to ensure rapid diffusion of the drug within the tissue in a sufficient concentration to trigger a response. Typical *in vivo* voltammograms recorded before and after the exposure to nomifensine exhibit an oxidation peak at ∼ 0.2 V, consistent with the oxidation potential of dopamine (Figure 6A). The nomifensine treat-

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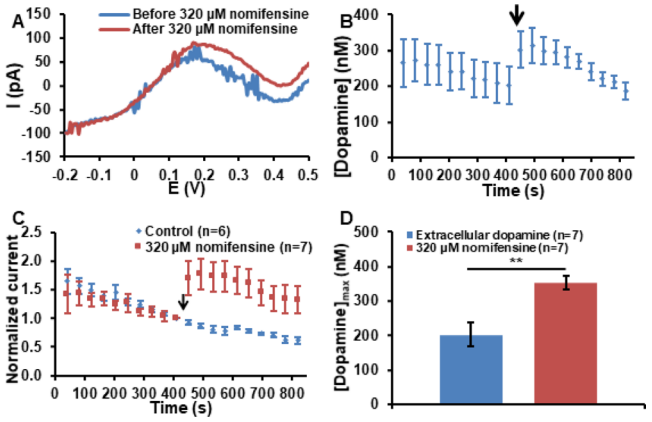

Figure 6. (A) Typical DPVs recorded at the implantation site in a 5 dpf zebrafish embryo exhibit an oxidation peak at 0.2 V associated with the oxidation of dopamine. After the addition of nomifensine in the near proximity of the embryo, the dopamine oxidation peak is increasing. (B) Average dopamine concentration−time trace measured in the tectum of 5 dpf zebrafish embryos. The arrow indicates the injection of nomifensine in the near proximity of the embryos at 410 s. The error bars represent the standard error of the mean for n = 7 replicate experiments in individual embryos. (C) Comparison of normalized oxidation current−time traces for control and nomifensine addition experiments. The oxidation currents were normalized to the value measured just before the addition of the control or nomifensine sample, as indicated by the arrow. The error bars represent the standard error of the mean for "n" replicate experiments in individual embryos. (D) Comparison between the average dopamine concentrations measured before and after the addition of nomifensine. The extracellular dopamine concentration represents the average concentration measured just before the injection of nomifensine in the medium. The dopamine concentration associated with the exposure to 320 μM nomifensine is the average of the maximum dopamine concentrations after the injection of nomifensine for each single measurement. The error bars represent the standard error of the mean for n = 7 replicate experiments in individual embryos. Statistical significance is calculated using the paired t-test and it is indicated by one (*) and two (**) asterisks at $p < 0.05$ and $p < 0.01$, respectively.

ment leads to an increase in the dopamine oxidation peak. The average dopamine concentration−time trace recorded from multiple embryos (n = 7) shows an immediate increase in the dopamine concentration right after the exposure to nomifensine (Figure 6B). The increased dopamine levels remained relatively consistent until the end of the measurement. To better visualize the current profile difference in comparison with the control experiment mentioned above, the oxidation currents were normalized for the control and nomifensine exposure measurements (Figure 6C). The current−time traces show a significantly different profile after the injection of control and nomifensine samples, with an approximatively twofold current increase at 0.2 V for nomifensine. The comparison between the extracellular dopamine concentration before drug addition and the maximum dopamine concentration observed after nomifensine exposure indicates a significant increase in synaptic dopamine as expected (Figure 6D). The extracellular dopamine concentration measured at 201.9 (±34.9) nM increases by ∼75%, up to 352.2 (±20.0) nM, after the injection of nomifensine in the near proximity of the embryo ($p < 0.01$, n = 7 zebrafish embryos). The post-use assessment of microelectrodes in the presence of dopamine confirms the robustness of the pretreated CFMEs for *in vivo* measurements (Figure S5).

To verify if norepinephrine has any contribution to the observed analytical signal, desipramine was used to treat embryos during electrochemical measurements. Desipramine is a selective norepinephrine uptake inhibitor, leading to increased norepinephrine accumulation in the extracellular space in the brain.[58] The drug was applied in an excess concentration in the near proximity of the zebrafish embryo. The typical voltammograms recorded at the implantation site before and after the application of desipramine do not show a noticeable difference in the oxidation peak intensity (Figure 7A). The time-dependent average dopamine profile for n = 7 individual embryos does not show major changes after the addition of desipramine (Figure 7B). The performance of the microelectrodes before and after *in vivo* measurements is maintained fairly well (Figure S6). The lack of a quantifiable effect during the exposure to desipramine is better seen when the normalized current−time traces for control and desipramine exposure experiments are compared (Figure 7C). Furthermore, the average dopamine concentration measured before and after the exposure to desipramine does not vary significantly (Figure 7D). The extracellular dopamine concentration measured initially at 369.6 (±29.6) nM only changes up to an average dopamine concentration of 382.4 (±51.3) nM after the exposure to desipramine (not significant for $p < 0.05$; n = 7 individual embryos). Therefore, the exposure of live zebrafish embryos to an excess concentration of desipramine does not have a statistically significant impact on the measured analytical signal.

In summary, the quantitative measurement of dopamine at the implantation site and further pharmacological manipulation confirmed the selectivity of measurements and provided the first quantitative values of the dopamine level in the tectum of 5 dpf zebrafish embryos. Although serotonin is expected to have higher oxidation potentials and it is therefore excluded, dopamine and norepinephrine are discriminated against by the differential behavior observed in the presence of nomifensine and desipramine. Nomifensine has been shown to function as a potent inhibitor of both the dopamine and norepinephrine transporters,[59] while desipramine specifically binds to norepinephrine and serotonin transporters.[58] The significant increase in the electrochemical signal at 0.2 V in the presence of nomifensine, but not desipramine, suggests that the detected analyte is indeed dopamine. The measured dopamine concentrations in 5 dpf embryos are in the same range as the values reported for the adult zebrafish whole brain and brain slices.[22] Slightly lower values are expected as the embryos used in this study are at an early developmental stage. The observed variation of the dopamine concentration under the influence of nomifensine reported here also follows a similar pattern to previous reports. Shang *et al.* have reported a twofold increase in the electrochemical signal specific to dopamine oxidation; however, they have not provided a quantitative value for the dopamine content.[25] Similarly, a twofold dopamine concentration increase has been observed in the whole brain and in brain slices treated with nomifensine.[22] Overall, our results suggest that the developed microelectrodes can be successfully used to achieve the time-dependent monitoring of dopamine in the tectum region of the mesencephalon in live 5 dpf zebrafish embryos and confirm the usefulness of these measurements for studying the effect of pharmacological interventions on dopamine levels using conventional drugs.

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ACS Meas. Sci. Au 2022, 2, 261−270

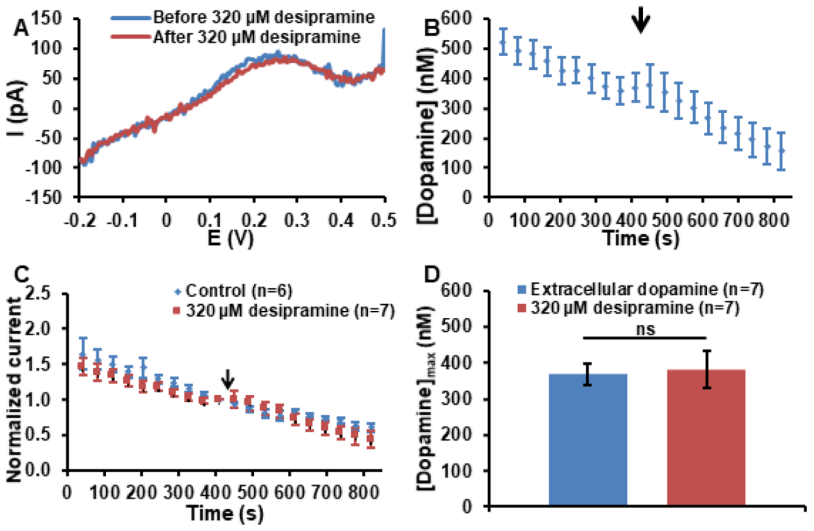

Figure 7. (A) Typical DPVs recorded at the implantation site in a 5 dpf zebrafish embryo exhibit an oxidation peak at 0.2 V associated with the oxidation of dopamine. After the addition of desipramine in the near proximity of the embryo, the dopamine oxidation peak intensity does not show a significant change. (B) Average dopamine concentration−time trace measured in the tectum of 5 dpf zebrafish embryos. The arrow indicates the injection of desipramine in the near proximity of the embryos at 410 s. The error bars represent the standard error of the mean for n = 7 replicate experiments in individual embryos. (C) Comparison of normalized oxidation current−time traces for control and desipramine addition experiments. The oxidation currents were normalized to the value measured just before the addition of the control or desipramine sample, as indicated by the arrow. The error bars represent the standard error of the mean for "n" replicate experiments in individual embryos. (D) Comparison between the average dopamine concentrations measured before and after the addition of desipramine. The extracellular dopamine concentration represents the average concentration measured just before the injection of desipramine in the medium. The dopamine concentration associated with the exposure to 320 μM desipramine is the average of the maximum dopamine concentrations after the injection of desipramine for each single measurement. The error bars represent the standard error of the mean for n = 7 replicate experiments in individual embryos. Statistical significance is calculated using the paired t-test and it is indicated by "ns" for no statistical significance.

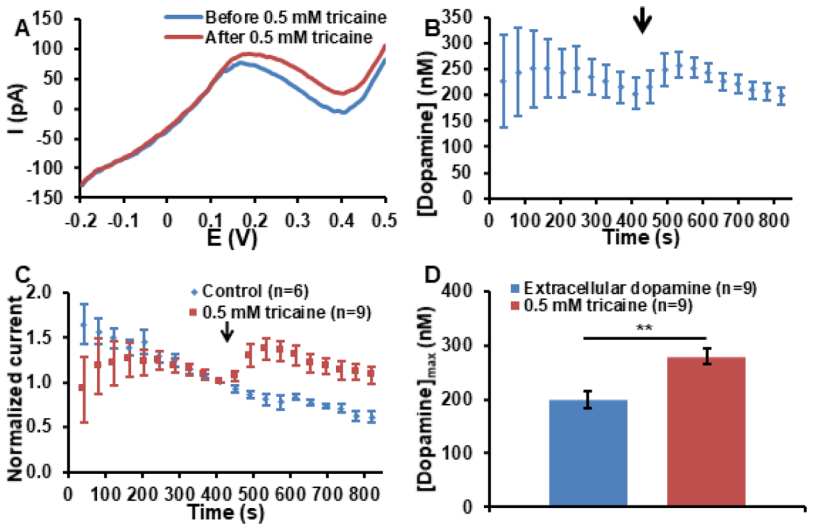

Figure 8. (A) Typical DPVs recorded at the implantation site in a 5 dpf zebrafish embryo exhibits an oxidation peak at 0.2 V associated with the oxidation of dopamine. After the addition of tricaine in the near proximity of the embryo, the dopamine oxidation peak is increasing. (B) Average dopamine concentration−time trace measured in the tectum of 5 dpf zebrafish embryos. The arrow indicates the injection of tricaine in the near proximity of the embryos at 410 s. The error bars represent the standard error of the mean for n = 9 replicate experiments in individual embryos. (C) Comparison of normalized oxidation current−time traces for the control and tricaine addition experiments. The oxidation currents were normalized to the value measured just before the addition of the control or tricaine sample, as indicated by the arrow. The error bars represent the standard error of the mean for "n" replicate experiments in individual embryos. (D) Comparison between the average dopamine concentrations measured before and after the addition of tricaine. The extracellular dopamine concentration represents the average concentration measured just before the injection of nomifensine in the medium. The dopamine concentration associated with the exposure to 0.5 mM tricaine is the average of the maximum dopamine concentrations after the injection of tricaine for each single measurement. The error bars represent the standard error of the mean for n = 9 replicate experiments in individual embryos. Statistical significance is calculated using the paired t-test and it is indicated by one (*) and two (**) asterisks at $p < 0.05$ and $p < 0.01$, respectively.

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Brain Dopamine Dynamics in Zebrafish Embryos Exposed to the Anesthetic Agent

The use of chemical agents to induce analgesia, sedation, or anesthesia is prevalent in multiple zebrafish assays.[60] The use of anesthetic agents on embryos generally induces a stress state followed by a stress response. A typical stress response is the increased release of neurochemicals in the nervous system.[61] Tricaine (or MS-222) is one of the common anesthetic agents employed in a range of procedures on embryonic and larval zebrafish.[62] To quantify the effects of anesthetic agents on neurophysiology, embryos were exposed to tricaine and the dopamine concentrations in the tectum were determined electrochemically.

Typical voltammograms recorded in the tectum before and after the exposure to tricaine show a distinct increase in the electrochemical current associated with dopamine oxidation (Figure 8A). The average dopamine concentration−time profile recorded for n = 9 individual embryos indicates a slow increase in dopamine release, with a maximum observed at ∼ 100 s after the injection of tricaine in the near proximity of the embryo (Figure 8B). It is interesting to note that the increased levels of dopamine following tricaine exposure are relatively short lived. The concentration rapidly decreases to almost normal levels after 300 s. The normalized current−time traces comparison between the control and tricaine addition experiments shows a clear change in oxidation currents at ∼ 0.2 V associated with stress-induced catecholamine release (Figure 8C). In terms of absolute values, the average extracellular dopamine concentration of 198.6 (±15.7) nM increases to a maximum of 278.8 (±14.0) nM after the exposure to 0.5 mM tricaine, representing a significant increase of more than 40% ($p < 0.01$, n = 9 zebrafish embryos) (Figure 8D). In comparison with the previous experiments, the CFMEs decreased selectivity in the post-calibration tests (Figure S7).

To our knowledge, this is the first report of a quantitative assessment of the time-dependent effect of exposure to an anesthetic on the neurochemical activity of live zebrafish embryos. The tricaine concentration employed here was reported to induce only a paralysis effect and not lead to euthanasia.[63] However, its effect on the brain's neurochemical dynamics is still substantial. Tricaine acts through the inhibition of the Na^+ ion channels, leading to the suppression of the nervous system.[64] Therefore, the increase in extracellular dopamine suggests there is an indirect effect of tricaine on the nervous system. The exposure to anesthetics triggers a stress response consisting of increased concentrations of catecholamines and corticosteroids.[65,66] Induction of stress is associated with the activation of the hypothalamus−pituitary−adrenal (HPA) axis.[67] The existence of common neuronal pathways between the hypothalamus and optic tectum could induce the stimulation of dopamine release in the optic tectum as an indirect consequence of the stress response.[28,68] The relatively short duration of increased dopamine levels observed in our research is consistent with studies reporting that the concentration of catecholamines released as a result of acute stress drops rapidly.[65] Another potential explanation is related to the ability of fish to detect the anesthetic agent early through taste, smell, or skin irritation.[65] Dopamine release in the optic tectum could be the results of olfactory stimulation with tricaine, as it was observed before with other olfactory stimulants.[25] Assessing the magnitude of the catecholamine stress response in zebrafish is important, as the dopamine pooling in the brain could interfere with assays where tricaine is employed as a sedative or anesthetic by increasing the levels of measurable extracellular dopamine.[69] *In vivo* electrochemistry can monitor analytes in zebrafish embryos to determine neurochemical changes resulting from sedation. Future improvement of the temporal resolution by varying the DPV parameters could lead to a better assessment of the release and uptake segments of the concentration curve.

CONCLUSIONS

We have demonstrated the successful time-dependent detection of dopamine in the tectum of live 5 dpf zebrafish embryos. An electrochemically pretreated carbon fiber microsensor has been developed to measure dopamine with improved sensitivity in low ionic strength environments. We have shown that the mild overoxidation of carbon fibers in a basic solution leads to improved performance for the detection of dopamine in particular, but not serotonin. The use of repetitive scan DPV allowed us to differentiate between analytes oxidizing at close oxidation potentials while also acquiring data with sufficient time resolution for the detection of extracellular dopamine. Using this approach, the extracellular dopamine levels in the tectum of 5 dpf zebrafish embryos and *in vivo* pharmacological manipulation using conventional drugs were quantitatively measured. The differential behavior of the measured electrochemical signal when the embryos are under the influence of nomifensine or desipramine suggests specific detection of dopamine at the implantation site. Furthermore, we have quantified the effect of tricaine, a conventional anesthetic used in zebrafish assays, on the neurochemical profile in embryos. The exposure to tricaine leads to a stress response seen as a temporary increase in dopamine levels. Although more research is needed to explore this model, this work demonstrates that electrochemical measurements in zebrafish during embryogenesis provide an *in vivo* model system for studying neurological mechanisms and drug-screening applications. Furthermore, the application of this technology can provide a greater understanding of the role of dopamine and potentially other neurotransmitters in the brains of zebrafish and other vertebrate models.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmeasuresciau.1c00051.

> Comparison of the voltammetric response recorded before and after the alkaline pretreatment of CFMEs for repetitive measurements of 1 μM dopamine, repetitive scan DPVs for the measurement of 1 μM dopamine using a cleaning step, and pre-calibration and post-calibration data in the presence of 1 μM dopamine for all the *in vivo* measurements (control and exposure to nomifensine, desipramine, and tricaine) (PDF)

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E.D., A.D., K.N.W., and S.A. conceived the experiments. E.D. and A.D. carried out the experiments. E.D., A.D., K.N.W., and S.A. wrote the manuscript.

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Notes

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