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Voltammetric Detection of 5-Hydroxytryptamine Release in the Rat Brain

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Abstract

5-HT is an important molecule in the brain that is implicated in mood and emotional processes. *In vivo*, its dynamic release and uptake kinetics are poorly understood due to a lack of analytical techniques for its rapid measurement. Whereas fast-scan cyclic voltammetry with carbon fiber microelectrodes is used frequently to monitor sub-second dopamine release in freely-moving and anesthetized rats, the electrooxidation of 5-HT forms products that quickly polymerize and irreversibly coat the carbon electrode surface. Previously described modifications of the electrochemical waveform allow stable and sensitive 5-HT measurements in mammalian tissue slice preparations and in the brain of fruit fly larvae. For *in vivo* applications in mammals, however, the problem of electrode deterioration persists. We identify the root of this problem to be fouling by extracellular metabolites such as 5-HIAA, which is present in 200-1000 times the concentration of 5-HT and displays similar electrochemical properties, including filming of the electrode surface. To impede access of the 5-HIAA to the electrode surface, a thin layer of Nafion®, a cation exchange polymer, has been electrodeposited onto cylindrical carbon-fiber microelectrodes. The presence of the Nafion® film was confirmed with environmental scanning electron microscopy and was demonstrated by the diminution of the voltammetric signals for 5-HIAA as well as other common anionic species. The modified microelectrodes also display increased sensitivity to 5-HT, yielding a characteristic cyclic voltammogram that is easily distinguishable from other common electroactive brain species. The thickness of the Nafion® coating and a diffusion coefficient (D) in the film for 5-HT were evaluated by measuring permeation through Nafion®. *In vivo*, we used physiological, anatomical and pharmacological evidence to validate the signal as 5-HT. Using Nafion®-modified microelectrodes, we present the first endogenous recording of 5-HT in the mammalian brain.

Keywords

Serotonin; 5-HT; Electrochemistry; Microelectrodes; 5-HIAA; Electrodeposition; Nafion; *In-vivo*; Fast-scan Cyclic Voltammetry

INTRODUCTION

5-Hydroxytryptamine (5-HT), or serotonin, is an electroactive indole that acts as an important neurotransmitter in the brain. It has been the focus of considerable research efforts over the last 30 years. Despite its implication in several neurological disorders such as depression and

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anxiety,[1] for which serotonin-altering medications are widely prescribed, little is known about its dynamics in the brain. Limitations in analytical techniques for *in vivo* 5-HT detection come from a combination of low endogenous levels and rapid uptake of 5-HT from the extracellular space.

Existing electrochemical methods such as chronoamperometry and differential pulse voltammetry have provided the scientific community with important information about 5-HT uptake and information about 5-HT levels.[2,3] However, these techniques are limited in their selectivity, and identification of a signal as 5-HT can only be confirmed by measuring uptake of exogenous 5-HT injections. Fast-scan cyclic voltammetry would provide the temporal resolution as well as selectivity necessary to record endogenous release and uptake, and the use of this technique for 5-HT would enable researchers to fill a large gap in the literature.

We have previously reported on the use of fast-scan cyclic voltammetry for *in vivo* dopamine release and uptake monitoring.[4,5] Both dopamine and 5-HT undergo reversible 2-electron oxidations that yield characteristic and distinguishable cyclic voltammograms. However, unlike dopamine, the oxidative electrochemistry of 5-HT is complex, yielding many oxidation side-products which adsorb to the carbon surface on the conventional positive sweeping scan.[6,7] Even at the high scan rates (400 Vs^{-1}) used to detect dopamine with fast-scan cyclic voltammetry, these oxidation side-products still polymerize and produce films that quickly and irreversibly foul the carbon surface. This fouling has been reported to be minimized on diamond microelectrode surfaces since the diamond surface expresses significantly fewer oxygen groups when compared to the carbon surface.[8] These microelectrodes have been used to provide real-time monitoring of 5-HT in guinea pig ileum where the endogenous 5-HT levels are relatively high (10-20 μM).[8,9] Neurochemical measurements with diamond microelectrodes, however, remain a challenge in part due to the relatively large size of the microelectrodes (~76 μm diameter). [10] 76 μm is larger than inter-capillary distance in the rat brain (~30 μm); in brain tissue, such devices with dimensions larger than inter-capillary distance have been shown to cause tissue damage.[11,12] Even modest tissue damage caused by these microelectrodes may impede the measurements of synaptic 5-HT overflow that carbon fiber microelectrodes (~5 μm) are able to detect.

In previous work, we modified the electrochemical waveform that is routinely used for dopamine monitoring with carbon fiber microelectrodes to minimize the majority of side reactions using Nafion®-modified carbon-fiber disc microelectrodes.[13] We showed the feasibility of this modification for *in vivo* use via a model described by Stamford *et al.*, where striatal dopaminergic cells are forced to release high levels of 5-HT by arresting dopamine production and pre-loading dopamine neurons with a 5-HT precursor (5-HTP).[14] Although this was not a physiological situation, it demonstrated that fast-scan cyclic voltammetry can be applied to 5-HT measurements. This modified waveform also enables stable detection of 5-HT in tissue slice preparations in experiments lasting 8 hours and in experiments with *D. melanogaster*.[15,16,17] However, despite these studies, there remain no *in vivo* reports of the use of this approach for endogenous 5-HT detection.

A major physiological difference between tissue slice preparations and the intact brain is the concentration of extracellular metabolites such as 5-hydoxyindole acetic acid (5-HIAA). This metabolite is present at 200-1000 times the basal concentration of 5-HT in serotonin containing regions in the intact brain whereas in tissue slices, extracellular species such as ascorbic acid and 5-HIAA wash out.[18,19,20,21] 5-HIAA is an indole with electrochemical oxidation properties similar to 5-HT. Given the high and persistent baseline extracellular concentrations of 5-HIAA, we hypothesize that *in vivo* microelectrode implantation in a 5-HT rich brain region, even in the absence of 5-HT release, will lead to a quick and profound deterioration of the microelectrode due to electrode fouling by 5-HIAA.

Because 5-HIAA is negatively-charged in solution, cation exchange polymers such as Nafion® can be deposited onto the microelectrode surface to limit its access. Nafion® has been extensively used as a surface modification polymer to repel interferents and foulants while exerting a cationic preconcentration effect that leads to increases in sensitivity for positively-charged anions in electrochemical applications. [3,22,23,13,24,25] Carbon-fiber microelectrode discs can be dip-coated with Nafion® in a straightforward process due to electrostatic properties of silica groups on the surface of the glass capillary.[13,25] However, the small surface area of the discs makes them unsuitable for *in vivo* use: the microelectrode must have a larger surface area to sample enough release sites to record a substantial signal since these sites are not uniformly distributed throughout the tissue. Cylindrical carbon-fiber microelectrodes provide a large enough surface area to address this problem but uniform deposition of Nafion® onto cylindrical carbon-fiber microelectrodes is more challenging because the carbon fibers are inherently hydrophobic, and thus repel the negatively-charged polymer. Successful dip-coating of cylindrical paste microelectrodes often leads to thicker Nafion® films of 1-3 μm, which in application to fast-scan cyclic voltammetry could lead to significant increases in response time. [24] Electrodeposition of the negatively-charged Nafion® polymer allows for more control over the thickness of the film. [22,26] When used in combination with differential pulse voltammetry, high deposition potentials (3V) can rapidly deposit Nafion® uniformly on the carbon surface [22]. However, these potentials cause significant changes to the carbon surface, making it highly absorptive, which when combined with fast-scan cyclic voltammetry could also lead to increases in response time. [27,28]

In this paper, we modify a technique described by Rice *et al.* to electrochemically deposit a thin, uniform layer of Nafion® onto a cylindrical carbon-fiber microelectrode.[26] Environmental scanning electron microscopy (ESEM) imaging displays the Nafion® film on the carbon fiber surface and i*n vitro* analyses confirm an attenuation of diffusible 5-HIAA to the microelectrode surface. Furthermore, the Nafion® coating increases the sensitivity of the microelectrode to 5-HT. The cyclic voltammogram can easily be distinguished from other electroactive brain species, and common anionic species such as ascorbic acid are repelled. In previous work, we showed that microelectrodes with Nafion® films of 340 nm still allowed rapid enough time responses to observe *in vivo* chemical dynamics.[29] In this work, we modify the experimental deposition parameters to obtain a similar thickness. Using an *in vitro* dopamine step response and the diffusion coefficient of dopamine through Nafion®, we calculate the thickness of the elecrodeposited Nafion® film. Further, we back-calculate a diffusion coefficient for 5-HT through Nafion®.

To validate this modified technique *in vivo*, we apply the microelectrodes to a rat model of 5-HT release in which we electrically stimulate 5-HT cell bodies in the dorsal raphe nucleus (DRN), and measure their terminal output in the substantia nigra reticulata (SNR). The efficacy of the technique is verified by addressing previously described criteria for validation of microsensor selectivity *in vivo*. [30] These include voltammetric identity, anatomical and physiological verification, chemical validation of 5-HT presence in release sites and pharmacological validation.

EXPERIMENTAL SECTION

Carbon-fiber Microelectrodes

Carbon-fiber microelectrodes were constructed as described previously.[31] Cylindrical microelectrodes were made by vacuum aspiration of single a 2.5-μm-radius T-650 carbon fiber (Thornel, Amoco Co.) into a glass capillary of 0.6 mm external diameter and 0.4mm internal diameter (A-M Systems, Inc., Sequim, WA). A micropipette puller (Narashige, Tokyo, Japan) was used to taper the glass and form a carbon-glass seal. The exposed length of the carbon fiber was cut to approximately 100 μm.

Nafion® Electrodeposition

The exposed length of carbon fiber from the glass tip of the microelectrode was soaked for 30 minutes in isopropyl alcohol (IPA) to clean the surface.[32] The procedure for electrodeposition of Nafion® was modified from work by Rice *et al.* 26 The tip of microelectrode was lowered in Nafion® solution (5 wt% 1100 EW NAFION ® in methanol, ION POWER, DE) and a constant potential of + 1.0 V vs. Ag/Ag Cl was applied to the microelectrode surface for 30 seconds. The microelectrode was dried in air for 10 seconds and then at 70° C for 10 minutes. The microelectrodes were stored dry prior to use.

Environmental Scanning Electron Microscopy (ESEM)

ESEM allows for imaging of low conductance or entirely non-conductive samples while eliminating the need for sputter coating. Control and Nafion®-modified microelectrodes were imaged in low-vacuum (0.65 Torr) on a FEI Quanta 200 ESEM equipped with Schottky field emission gun (accelerating voltage used in the studies was 10 kV); secondary electron images were obtained with a large field secondary electron detector.

Data Acquisition

In most experiments, a 5-HT specific waveform was used as described elsewhere [13]. Briefly, this waveform was applied with a scan rate of 1000 V s^{-1} with a resting potential of 0.2 V versus Ag/AgCl. The potential is ramped up to 1.0 V, then down to -0.1 V and back to resting potential of 0.2 V. In one experiment, the conventional dopamine waveform was used as described by Heien *et al.* 33 In brief, this triangular waveform was applied at 400 V s^{-1} with a resting potential of -0.4V. The potential is then ramped up to 1.3V and back to the resting potential of -0.4 V. In both cases, scans were repeated every 100 ms. A customized version of TH-1 software (ESA, Chelmsford, MA) written in LABVIEW (National Instruments, Austin, TX) was used for waveform output and data acquisition. The software allowed output of waveforms with a DAC/ADC card (NI 6251 M). The second card (NI 6711) was used for triggering the DACs and ADCs as well as for synchronization of the electrochemical experiment with flow injection of the analytes. A custom-built instrument for potential application to the electrochemical cell and current transduction (University of North Carolina at Chapel Hill, Department of Chemistry Electronics Facility) was employed. Signal processing (background subtraction, signal averaging, and digital filtering (4-pole Bessel Filter, 5 kHz)) was software-controlled.

Chemicals

5-HT, 5-HIAA, dopamine, norepinephrine, epinephrine, DOPAC (3,4-dihydroxyphenylacetic acid), ascorbic acid, citalopram and GBR 12909 were purchased and used as received from Sigma-Aldrich. A physiological buffer solution (15 mM Tris, 126 mM NaCl, 2.5 mM KCl, 25 mM $NaHCO_3$, 2.4 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2.0 mM Na_2SO_4) at pH 7.4 was used in all flow injection analysis experiments. All aqueous solutions were made using doubly distilled deionized water (Megapure System, Corning model D2).

Flow Injection Apparatus

The flow injection analysis apparatus has been described elsewhere.[34] Briefly, the carbon-fiber microelectrode was affixed in the output of a flow-injection apparatus that consisted of a six-port HPLC loop injector mounted on a two-position actuator (Rheodyne model 7010 valve and 5701 actuator) that was operated with a 12 V DC solenoid valve kit (Rheodyne, Rohnert Park, CA). The apparatus allowed for the introduction of a rectangular pulse of analyte to the microelectrode surface by a syringe infusion pump (Harvard Apparatus model 940, Hollison, MA) at a flow rate of 2 mL min^{-1}.

Modeling of Film Thickness and Diffusion Coefficients

A procedure from Kawagoe and Wightman was modified in order to determine both the thickness of the Nafion film, l_p, and the diffusion coefficient of 5-HT inside the Nafion film, $D_{5\text{-HT}}$. [29] The response to a step concentration of dopamine (10 μM) was measured on a Nafion-coated carbonfiber microelectrode using the modified 5-HT waveform in the flow injection system. The process was repeated with the same microelectrode for a step concentration of 5-HT (1 μM). Each peak current vs. time trace was extracted, corrected to zero background, and normalized to a maximum of 1. The normalized dopamine trace was fit to the equation:

$$\frac{C(t)}{C_0} = 1 - \frac{4}{\pi}\sum_{n=0}^{\infty}\frac{(-1)^n}{(2n+1)}\exp\left(-\frac{D\pi^2(2n+1)^2 t}{4l_p}\right) \quad \text{(Eq. 1)}$$

where C(t) is the concentration at the microelectrode surface, C_0 is the concentration in bulk solution outside the Nafion film, D is the diffusion coefficient of the analyte inside of the Nafion film, and t is the time from the initial exposure to the concentration bolus. l_p was allowed to vary between 1 and 1000 nm in 1 nm increments. A value of 1×10^{-9} cm^2s^{-1} (from previous work) was used for the diffusion coefficient for dopamine inside the Nafion film, D_{DA}, this value closely resembles the reported values through thick Nafion® films. [29,35] The value of l_p that gave the best least-squares fit between the model and the step concentration of dopamine was used as an initial estimate for the thickness of the Nafion film. This thickness was used in Equation 1 to fit the normalized response to the 5-HT concentration step where $D_{5\text{-HT}}$ was allowed to vary between 0.1×10^{-10} cm^2s^{-1} and 1.0×10^{-8} cm^2s^{-1} in 0.1×10^{-10} cm^2s^{-1} steps. The value of $D_{5\text{-HT}}$ that gave the best least-squares fit between the model and the step concentration of 5-HT was used to generate an improved estimate for l_p which was then used to obtain an improved estimate for $D_{5\text{-HT}}$ and the process was repeated iteratively until the solutions for l_p and $D_{5\text{-HT}}$ converged.

Surgery

Male Sprague-Dawley rats weighing between 350-425 grams were anaesthetized with urethane (1.5 g/kg rat weight) and positioned into a stereotaxic frame. Holes were drilled in the skull according to stereotaxic coordinates referenced to bregma and taken from a stereotaxic atlas. [36] Placement of the carbon-fiber microelectrode was in the SNR (stereotaxic coordinates AP, -5.2; ML, +2.0; DV, -8.5) and the bipolar stimulating electrode in the DRN (AP -8.0; ML, 0.0; DV, -6.0). The Ag/AgCl wire that served as the reference electrode was implanted into the contralateral hemisphere. Biphasic stimulating pulses, 2 ms each phase, 300 μA each phase, were applied at 60 Hz for two seconds to evoke 5-HT release. Stimulation-evoked release was recorded during and after the stimulation, and selectivity for 5-HT was verified pharmacologically. Clearance of 5-HT from the extra-cellular fluid depends largely upon selective re-uptake through serotonin uptake transporters (SERTs), and these transporters can be blocked by selective serotonin reuptake inhibitors (SSRIs).[37] To pharmacologically verify our signal, we administered the SSRI citalopram (10 mg/kg).[37] Similarly, clearance of dopamine can be selectively blocked by administration of a dopamine transporter (DAT) inhibitor, GBR 12909;[38] we administered GBR 12909 (15 mg/kg) to ascertain contribution of dopamine to the signal. [5] Student's t-tests were performed on paired data sets, $p<0.05$ was taken as significant.

RESULTS AND DISCUSSION

Characterization of Cylindrical Microelectrodes with the 5-HT Modified Waveform

We have characterized 5-HT detection with the waveform employed by Jackson *et al. in* 1995 but with cylindrical carbon-fiber microelectrodes without Nafion® coatings. Figure 1 shows *in vitro* comparisons between a single injection of either dopamine **(1 and 2)** or 5-HT **(3 and 4)** onto clean microelectrodes with (**A)** the conventional waveform used for detection of dopamine at 400 Vs^{-1} and (**B)** the modified Jackson waveform at 1000 Vs^{-1}. The color plots are a 2-dimensional representation of the cyclic voltammograms with current changes detected upon injection of the sample shown in false color.[39] Individual cyclic voltammograms recorded at three time points during the injection **(**1, 3 and 5 s) are displayed under each color plot. With the conventional waveform **(A)**, a typical dopamine response **(1)** shows the cyclic voltammograms are stable throughout the injection. In contrast, a 5-HT injection using this waveform **(3)** yields a cyclic voltammogram that changes at each time point, gaining extra peaks both on the forward and backward parts of the scan. This is indicative of the adsorption of products formed during the electrooxidation. This problem does not occur with the modified waveform for 5-HT **(4),** in which the cyclic voltammograms retain their shape throughout the injection as previously reported.[13] This is predominantly due to two characteristics of the modified waveform. First, when scanning at 1000 Vs^{-1} compared to 400 Vs^{-1}, the kinetics of the majority of the side reactions are too slow to have an effect. Second, by holding the rest potential of the microelectrode at 0.2 V some of these products are oxidized and their contribution to the current during the scan is minimized. A further point of interest is that the modified waveform does not show as good sensitivity towards dopamine **(2),** yielding approximately 8 nA for a 10 μM dopamine injection compared to 20 nA for a 1 μM 5-HT injection. Further, the cyclic voltammogram for dopamine can be easily distinguished from 5-HT by its reduction peak, which occurs on the positive scanning part of the wave (shown in red arrows). Dopamine reduction ordinarily occurs at -0.2V, which is beyond the negative sweep of the modified waveform. The reduction peak on the forward part of the negative scan is most likely due to the reduction of the quinone group on dopamine-o-quinone, a reaction that at 1000 Vs^{-1} is kinetically sluggish.

Microelectrode Fouling by 5-HIAA

Despite the fact that the modified 5-HT waveform has previously been implemented for recordings in *D. melanogaster*,[16] tissue slice preparations,[15,17] and *in vivo* models with preloaded dopaminergic cells,[13] we found that the electrochemical signal deteriorated during *in vivo* endogenous 5-HT monitoring. In preliminary *in vivo* investigations with the 5-HT waveform, we stimulated 5-HT release by DRN stimulation and measured in the SNR. In contrast to the Jackson study where 5-HT release was evoked artificially in a brain region that is physiologically dopaminergic, the SNR has high 5-HT content. In the SNR, the electrochemical responses were very low in amplitude and the cyclic voltammograms were irreproducible. The oxidation and reduction peaks observed on an *in vivo* cyclic voltammogram show little similarity to the *in vitro* chemical signature for 5-HT shown in Figure 1 (4). *In vivo*, a small oxidation peak is recorded on the initial positive scan, but a larger peak occurs on the subsequent negative scan. Additionally, the *in vitro* signal has a reduction peak at 0 V on the negative scan, whereas the *in vivo* cyclic voltammogram has this peak on the forward part of the negative scan (Figure 2 [A]**)**. The appearance of peaks on the reverse scans of *in vivo* signals indicates filming at the carbon fiber surface, which slows oxidative and reductive processes.

The results shown in Figure 1 (4) indicate that the filming is not due to 5-HT oxidation, since it does not occur with 5-HT alone in solution. The major metabolite of 5-HT in the brain, 5-HIAA, may contribute to this filming. It is present in the extracellular fluid in concentrations

that are approximately 200-1000 times that of 5-HT.[18] These two indoles are very similar in structure and may consequently have similar filming electrochemistry. Therefore, we added 5-HIAA (10 μM) to the flow injection buffer and the solutions of 5-HT. In this experiment, the microelectrode was cycled in the 5-HIAA containing buffer for two hours before injection. Cyclic voltammograms of 5-HT obtained under these conditions resemble those obtained *in vivo* (Figure 2 [B]**)**. Note that this fouling has not been observed in rat brain slices, wherein reproducible cyclic voltammograms can be obtained for up to 8 hours.[15,17] However, in slices, the tissue is continually perfused, allowing for washout of extracellular substances. For example ascorbate is rapidly lost from the perfused slices for this reason, and the same appears true for 5-HIAA.[19, 21, 26] This filming was not an issue in the Jackson *et al.* study, in which we recorded super-physiological concentrations of 5-HT release with a Nafion®-modified microelectrode disc.[13] Interestingly, MAO (monoamine oxidase), the enzyme responsible for 5-HIAA formation is profoundly less active in *D. melanogaster* than in mammalian nervous tissue, which minimizes the problem of 5-HIAA fouling in the fruit fly model.[40] We conclude that it is not feasible to obtain *in vivo* cyclic voltammograms that have the shape found *in vitro* in brain regions with high levels of 5-HT because of fouling of the microelectrode by 5-HIAA.

Exclusion of 5-HIAA from the Microelectrode Surface

5-HIAA is an anion at physiological pH. To exclude anions from carbon microelectrodes a cation exchange polymer is a popular choice.[22, 23, 25] Our own work has employed carbon-fiber microelectrode discs that can be coated with a thin layer (340 nm) of Nafion® by a dip-coating procedure.[5, 13, 29, 31, 41] Nafion® adsorbs readily to the silica groups of the glass barrel in which the microelectrode is housed. However, with a cylindrical microelectrode, the hydrophobic carbon fiber extends 75 - 100 μm beyond the glass barrel and is thus not proximate to these silica groups. Further, the dip-coating procedure does not uniformly coat the surface of the microelectrode. Because of its negative charge, Nafion® can be electrostatically deposited onto cylinders.[22, 26] We applied +1.0 V vs. Ag/Ag Cl to the cylindrical microelectrode while immersed in a 5% Nafion® solution in methanol. These parameters were chosen to provide protection against 5-HIAA while still maintaining a film sufficiently thin that the response time is not greatly increased. Figure 3 (i) shows ESEM images comparing **(A)** a bare cylinder to **(B)** a Nafion®-modified cylinder. The resolution of the instrument allows us to observe individual striations on the surface of the bare carbon-fiber microelectrode; however, because of electroformation of a film, these striations are indistinguishable on the Nafion®-modified carbon fiber. Also apparent is the excess extension of this film beyond the tip of the carbon fiber on the Nafion®-modified microelectrode.

Figure 3 (ii) shows a comparison between **(A)** a bare microelectrode to **(B)** a Nafion®-modified microelectrode in their responses to 1 um 5-HT (dashed line) and 10 μM 5-HIAA (solid line). Both microelectrodes measured approximately 100 μm in length. Before Nafion® coating, the sensitivity to 5-HT is about 30 times greater than for 5-HIAA. This is a result of the greater adsorption of 5-HT. Nafion® deposition reduces the 5-HIAA response at the microelectrode surface: in the displayed example, a bare microelectrode yields 10.7 nA in response to a 10 μM 5-HIAA injection, whereas a Nafion®-modified microelectrode yields a 3.30 nA response. Moreover, in this example, the Nafion®-modified microelectrode displays increased sensitivity to a 1 μM 5-HT injection, yielding 36.5 nA compared to the bare microelectrode response, 26.5 nA. On average, Nafion®-modified microelectrodes yield 49.5 ± 10.2 nA to a 1 μM 5-HT injection compared with 20.8 ± 1.84 nA for a bare electrode (n=4 ± SEM) (Table 1).

Exclusion of Electronegative Species and Survey of Other Endogenous Electroactive Neurotransmitters

Averaged responses (n=4) to common anionic and cationic species on a bare microelectrode compared to a Nafion electrodeposited microelectrode are presented in Table 1. We observe enhanced sensitivity to 5-HT and dopamine, sensitivity to norepinephrine and epinephrine remained approximately unchanged, and decreased sensitivity to the anions ascorbic acid, DOPAC, and 5-HIAA. These differences arise from the partition of the species between the Nafion® film and the solution.[42,24] Importantly, due to its high partition coefficient, the 5-HT molecule pre-concentrates heavily in the Nafion® membrane and results in the observed sensitivity enhancement, a phenomenon that has previously been reported to be advantageous for chemical sensors.[42] The increased sensitivity of Nafion®-modified microelectrodes is also evident in the averaged 5-HT calibrations for these two microelectrodes in a flow-through buffer that contains physiological levels of 5-HIAA (Figure 3 [iii]), where the fouling species are repelled. After Nafion® electrodeposition, the microelectrode is almost 200 times more sensitive to 5-HT than to 5-HIAA.

To confirm exclusion of other electroactive anions, we compared responses of bare and Nafion®-modified microelectrodes to other endogenous species. Figure 4 compares the responses of bare (grey) to Nafion®-modified (black) microelectrodes to **(i)** ascorbic acid (400 μM), **(ii)** DOPAC (20 μM), and **(iii)** 5-HIAA (10 μM). **A** shows the averaged responses to these species (n=4 ± SEM) while **B** shows a single example of the corresponding cyclic voltammograms for bare (grey, dashed) and Nafion®-modified (black, solid). The concentration ratios chosen are in the physiological range.[43, 44] It is evident that Nafion®-modified microelectrodes exclude the negatively-charged specie compared to bare microelectrodes. The ascorbic acid response is reduced from 50.6 ± 15.6 nA to 20.0 ± 4.70 nA. The DOPAC response is reduced from 4.9 ± 1.2 nA to 1.6 ± 0.2 nA. Finally, the 5-HIAA response is reduced from 13.8 ± 1.60 nA to 2.9 ± 0.9 nA. Thus, Nafion® electrodeposition enhances specificity for 5-HT by excluding anions.

Diffusion of 5-HT through a thin Nafion® Film

In our previous work, we modeled a dopamine step concentration response and calculated the thickness of a Nafion® dip-coated microelectrode disc to be 340 nm.[29] This thickness, despite slowing response time, still allows for observations of dynamic chemical changes associated with release and uptake of dopamine. We chose our electrodeposition parameters to attain this magnitude of thickness on our electrodeposited microelectrodes. Figure 5 shows a representative example application of Equation 1 in modeling step concentrations of dopamine and 5-HT in a flow injection system to determine l_p and $D_{5\text{-HT}}$. **A** shows simulations for various values of l_p to a normalized step concentration of dopamine, assuming a value of 1.0×10^{-9} cm^2s^{-1} for D_{DA} (from previous work), which is very close to the value reported for thick films (1.5×10^{-9} cm^2s^{-1}).[29,35] For the microelectrode shown in Figure 5, l_p is approximately 300 nm. Figure 5 (B) shows simulations for various values of $D_{5\text{-HT}}$ to a normalized step concentration of 5-HT, using l_p from the best fit model to the step in dopamine concentration. A value for $D_{5\text{-HT}}$ of approximately 5.0×10^{-10} cm^2s^{-1} best fits the experimental step in 5-HT concentration for this microelectrode. The value of $D_{5\text{-HT}}$ was used to improve the solution for l_p, which was used to improve the solution to $D_{5\text{-HT}}$ and the process was repeated until values for l_p and $D_{5\text{-HT}}$ converged. Values for l_p and $D_{5\text{-HT}}$ were determined to be 340 ± 8 nm and $7.6 \pm 0.8 \times 10^{-10}$ cm^2s^{-1}, respectively (average ± SEM, n=5). The value of l_p is consistent with values obtained by dip coating carbon-fiber microelectrode discs.[29] As expected, $D_{5\text{-HT}}$ and D_{DA} are both several orders of magnitude smaller than conventional values for diffusion coefficients in bulk solution because these molecules are diffusing through a denser polymer network. In addition, $D_{5\text{-HT}}$ is smaller than that of D_{DA}, which is consistent with values obtained previously in bulk solution.[45]

In Vivo Electrochemical Identification

To validate the *in vivo* signal, we address previously described criteria for characterization of a novel electrochemical signal.[30]

Figure 6 (i) compares the normalized cyclic voltammograms of 50 nM 5-HT *in vitro* to that obtained in SNR *in vivo*. Close similarity between the shapes of the cyclic voltammograms confirms identification of 5-HT *in vivo* ($r^2 = 0.94$). Figure 6 (ii) is the corresponding color plot from which this cyclic voltammogram is taken at the time indicated by the dashed white line.

In vivo microdialysis coupled to HPLC in the SNR confirms the release of 5-HT in the SNR brain region.[46,47,48] Tissue content analysis in the SNR confirms the presence of endogenous 5-HT at high levels.[49, 50]

In vivo investigations of DRN electrical stimulation have shown that stimulations even lower than 60 Hz are sufficient for evoking serotonin release.[51,52] Autoradiographic tracing studies report on the existence of a serotonergic pathway from the DRN to the SNR.[53-56] While there are no reports of a dopaminergic projection from the DRN to the SNR,[57] the target location of the microelectrode is proximal to dopamine terminals in a region just above the SNR, the substantia nigra pars compacta (SNC), which could theoretically contribute erroneously to the signal. Figure 6 (ii) demonstrates stimulation-evoked 5-HT release in the target area (8.5 mm ventral to the top of the skull) of the SNR. In 20 animals, we measured a stimulated release current of 0.63 ± 0.08 nA corresponding to a concentration of 12.7 ± 1.60 nM (n=20 ± SEM). This is consistent with previous reports of extracellular 5-HT levels as measured with differential pulse voltammetry and microdialysis.[3,58] Figure 6 (iii) demonstrates that little release is measured 1.5 mm above the SNR (7 mm ventral to the top of the skull), confirming specificity of the anatomical placement of the microelectrode.

Lastly, although we have shown that the modified microelectrode displays almost 50 times more sensitivity to 5-HT than to dopamine *in vitro*, we also verify pharmacologically that dopamine does not contribute to the *in vivo* signal. We administered GBR 12909 (15 mg kg^{-1}) and citalopram, (10 mg kg^{-1}) pharmaceutical agents that respectively inhibit re-uptake of dopamine by DAT or 5-HT by SERT.[38,37] If the transporter specific to the analyte is blocked, clearance time of the released specie will increase. Figure 7 shows stimulated release of 5-HT upon **(A)** control, **(B)** GBR 12909, and **(C**) citalopram treatments in the same anaesthetized rat displayed as current in 2-dimensional false color **(i)** and concentration vs. time traces **(ii)**. In 4 rats, GBR 12909 had no significant effect on release amplitude or clearance time, therefore the clearance of the analyte was not dependent on DAT. There is a notion in the 5-HT pharmacological literature that alterations in 5-HT reuptake may also change release dynamics: terminal 5-HT autoreceptors, which modulate release, have been shown to modulate uptake kinetics, presumably via interaction with SERTs. [2,59,60] Consistent with this, we found in 5 rats that citalopram significantly increased release amplitude from 13.1 ± 5.10 nM to 49.5 ± 13.9 nM (n=5 ± SEM, p=0.01) and more importantly, clearance time ($t_{1/2}$) from 1.70 ± 0.30 s to 5.20 ± 1.20 s (n=5 ± SEM, p=0.02), providing pharmacological identification of the released specie as 5-HT.

As we have confirmed the identity of the released analyte according to the criteria of Phillips and Wightman (2003),[30] we report that the presented technique enables the first quantitative, *in vivo* sub-second recording of 5-HT release and uptake.

CONCLUSION

Fast-scan cyclic voltammetry of *in vivo* 5-HT is challenging due to the electrochemically fouling side-products produced when 5-HT is oxidized. The electrochemical modifications that

allow 5-HT to be monitored with fast-scan cyclic voltammetry in tissue slice preparations and in *D. melanogaster* are not sufficient for *in vivo* monitoring where there are persistent and high levels of fouling 5-HT metabolites such as 5-HIAA. By electrodepositing Nafion® onto cylindrical carbon-fiber microelectrodes, we have shown that 5-HIAA can be excluded from the microelectrode surface; moreover, due to pre-concentration of the positively-charged 5-HT ions in solution, the microelectrodes are 50 - 100% more sensitive to 5-HT. Having reproduced the dip-coated thickness of the Nafion® film (~300 nm) described by our own previous work [29], we back-calculated a diffusion coefficient for 5-HT through a Nafion® film on a cylindrical carbon-fiber microelectrode. The Nafion®-modified cylinders significantly exclude contributions from common brain anions. Validation of the *in vivo* signal meets previously established criteria for identification of a novel analyte with a microsensor: we have demonstrated electrochemical validation, established independent chemical analyses, verified anatomical and physiological feasibility as well as pharmacological characterization of the signal.

We have used this technology to present the first measurement of 5-HT release and uptake *in vivo*. We plan to apply this method to answer long-anticipated questions regarding the dynamics, characteristics, and pharmaceutical effects of 5-HT *in vivo*.

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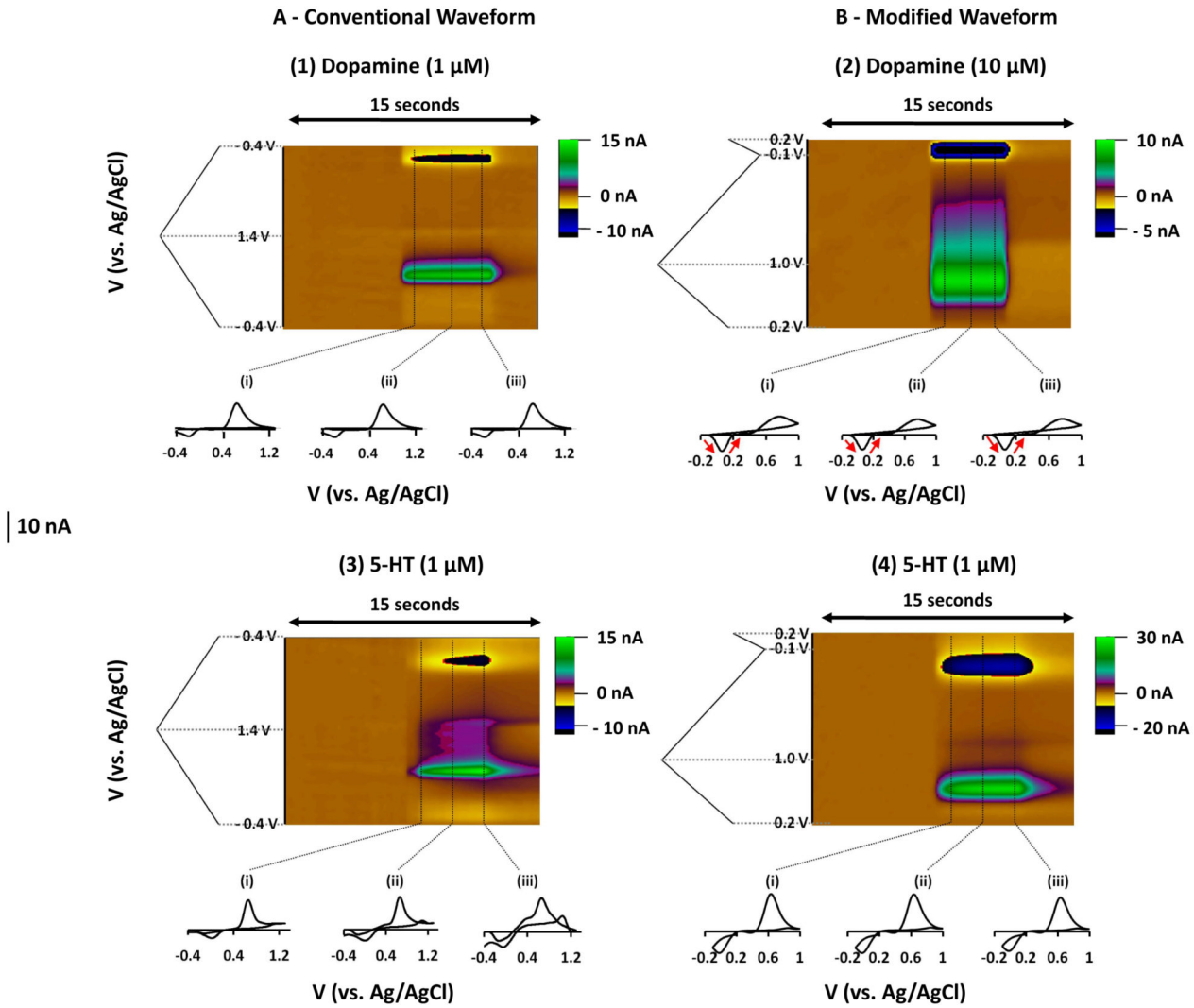

Figure 1.

Comparison of signals obtained following introduction of dopamine (1 and 10 µM) (top) and 5-HT (1 µM) (bottom) into a flow injection system by employing both the traditional **(A)** and modified **(B)** waveforms. The current response is represented in false color. Representative cyclic voltammograms (vs. Ag/Ag Cl) at time points **(i)**, **(ii)** and **(iii)** are single scans recorded at 1, 3, and 5 s post sample injection, respectively.

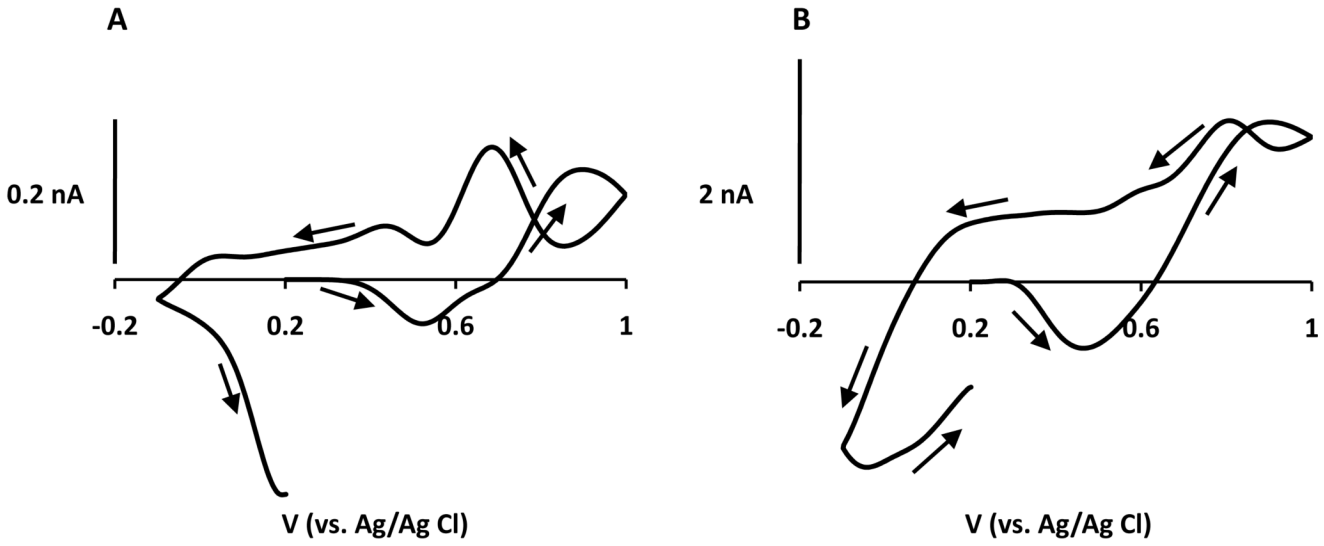

Figure 2.

Cyclic voltammograms (vs. Ag/Ag Cl) for **(A)** the signal obtained *in vivo* with DRN stimulation and SNR measurement and **(B)** *in vitro* signal obtained via 1 μM 5-HT injection onto the microelectrode after it had cycled for 2 hours in a buffer containing physiological levels of 5-HIAA (10 μm).

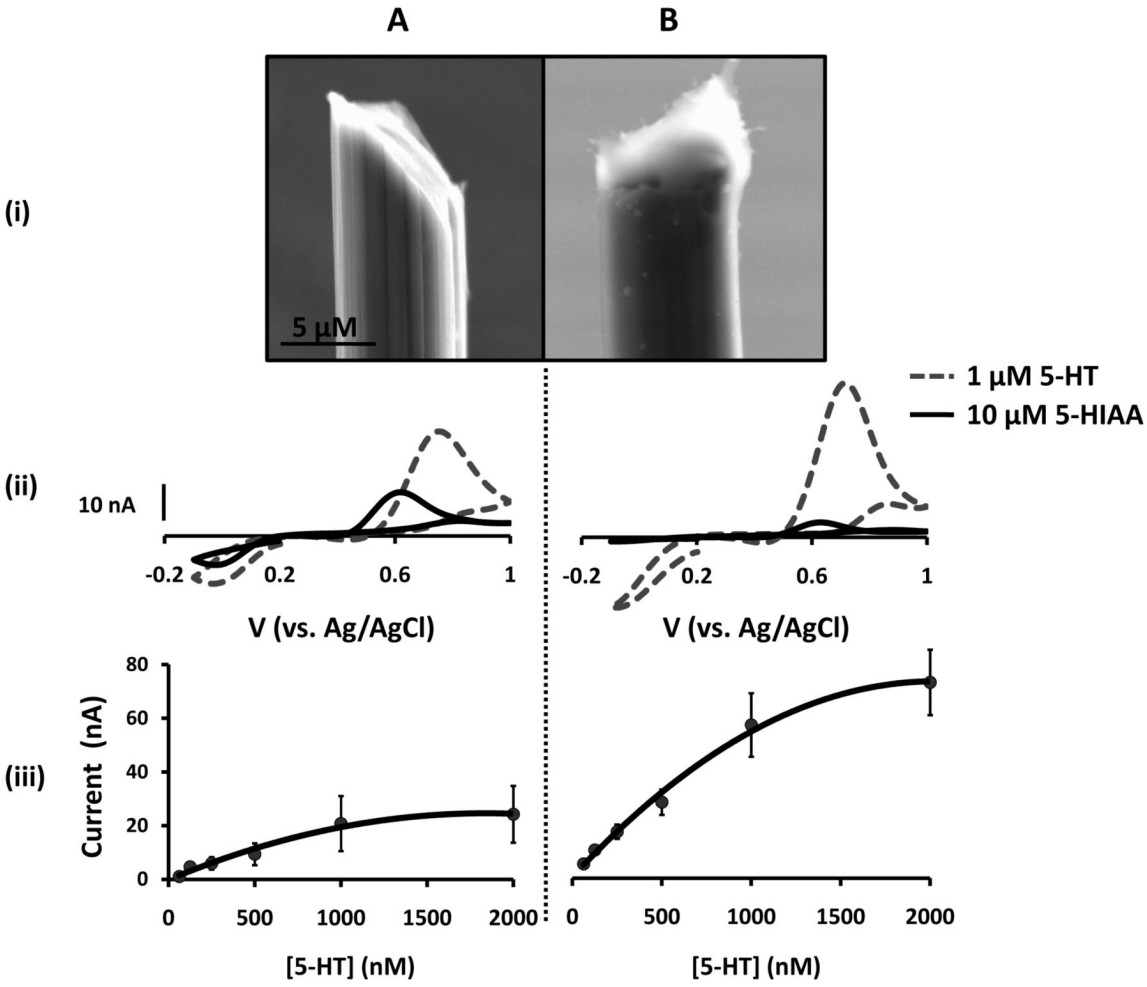

Figure 3.

Top panel **(i)** shows ESEM images for **(A)** bare microelectrode and **(B)** Nafion®-modified microelectrode. Panel **(ii)** shows cyclic voltammograms (vs. Ag/Ag Cl) for **(A)** a bare microelectrode compared to a **(B)** Nafion®-modified microelectrode and their responses to 10 µM 5-HIAA (black line) and 1 µM 5-HT (grey dashed line). Bottom panel **(ii)** shows the respective concentration vs. current calibrations of **(A)** a bare microelectrode and **(B)** a Nafion®-modified microelectrode in a buffer containing physiological levels of 5-HIAA (10 µM). The values are averages (n=4) ± SEM. The data is plotted with a polynomial line of best fit.

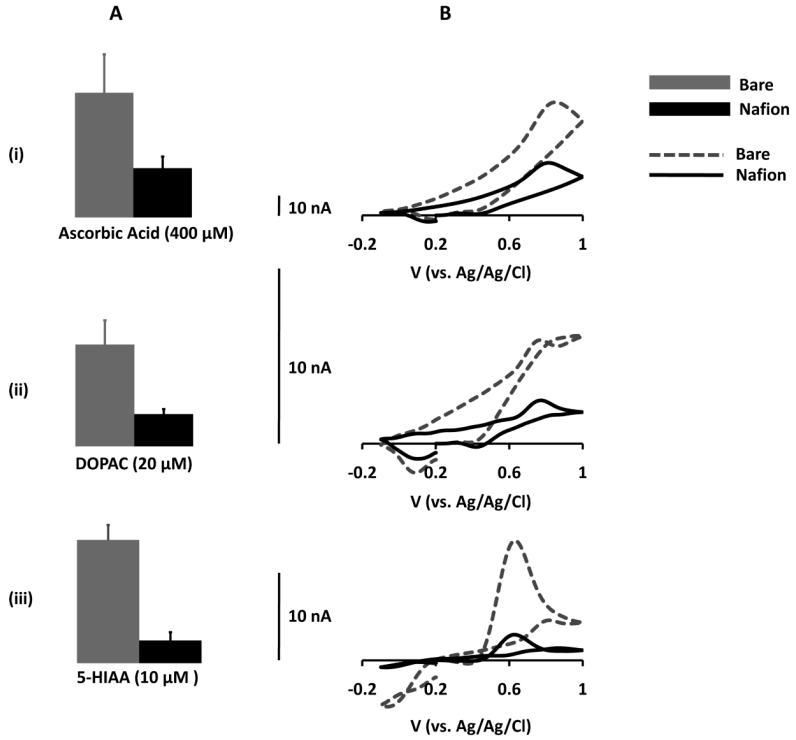

Figure 4.

Comparison of the responses of bare (grey) and Nafion®-modified (black) microelectrodes to electronegative species **(i)** ascorbic acid, **(ii)** DOPAC and **(iii)** 5-HIAA *in vitro*. The values are averages (n=4) ± SEM. **(B)** shows single examples of the cyclic voltammograms (vs. Ag/Ag Cl) for bare (dashed grey line) and Nafion®-modified (black solid line) for **(i)** ascorbic acid, **(ii)** DOPAC and **(iii)** 5-HIAA.

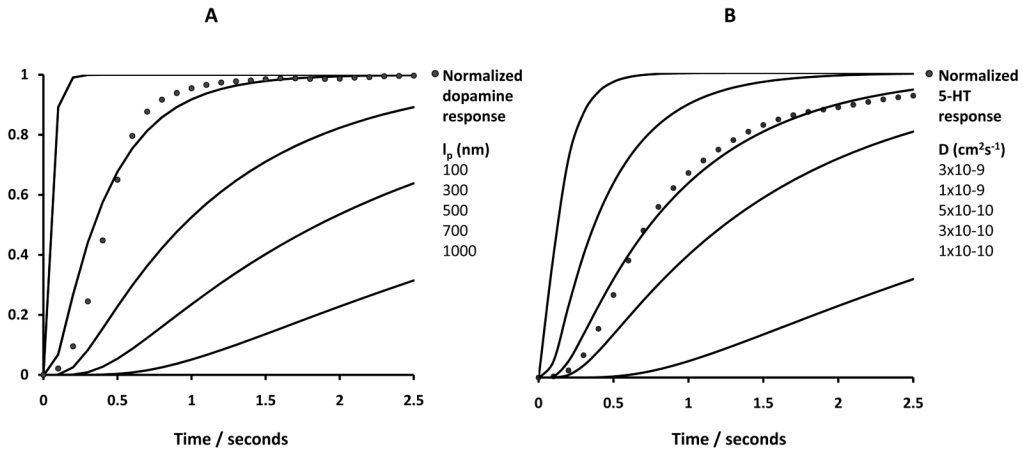

Figure 5.

Determination of l_p and $D_{5\text{-HT}}$ for a Nafion-coated carbon-fiber microelectrode using flow injection analysis. Panel **A** shows a normalized peak current response (grey dashed) towards a step concentration in dopamine (10 μM) fitted with equation 1 using various example values of l_p, assuming a value of D_{DA} of 1×10^{-9} cm^2s^{-1} (solid black lines). Panel **B** shows a normalized peak current response towards a step concentration of 5-HT (1 μM) fitted with equation 1 using various example values of $D_{5\text{-HT}}$ and a value of l_p obtained from the best fit to the dopamine step concentration.

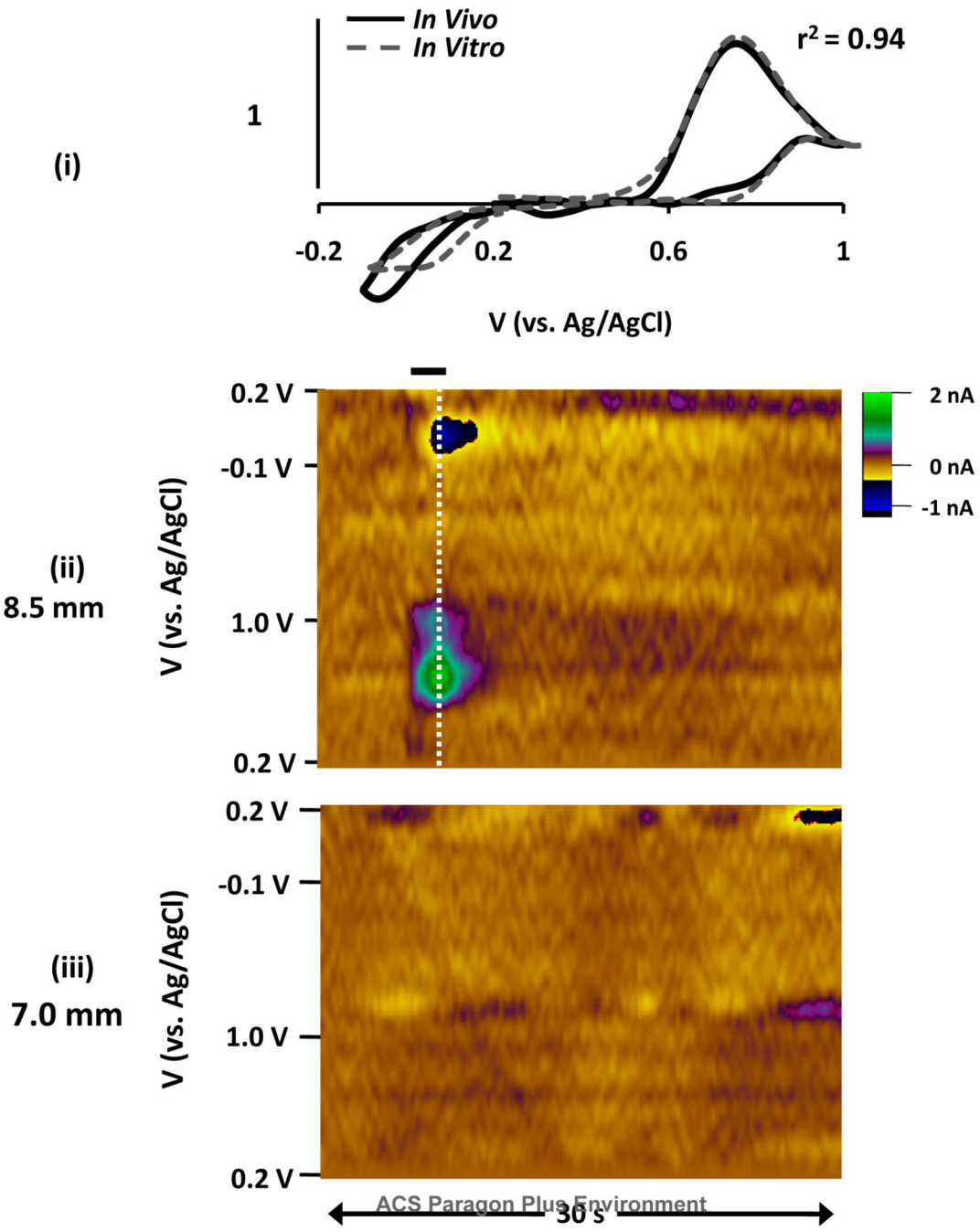

Figure 6.

Panel **(i)** shows a normalized comparison of an *in vivo* signal (black solid) to an *in vitro* 5-HT (100 nM) measurement. Panel **(ii)** is the corresponding *in vivo* color plot with potential on y-axis plotted against time on the x-axis at 8.5 mm ventral to the surface of the skull, the white dash line signifies the time section at which the cyclic voltammogram is taken. The current response is represented in false color, the black line horizontal to the x-axis is the duration of the stimulation. Panel **(iii)** is the *in vivo* color plot with potential on y-axis plotted against time on the x-axis at 7 mm ventral to the surface of the skull.

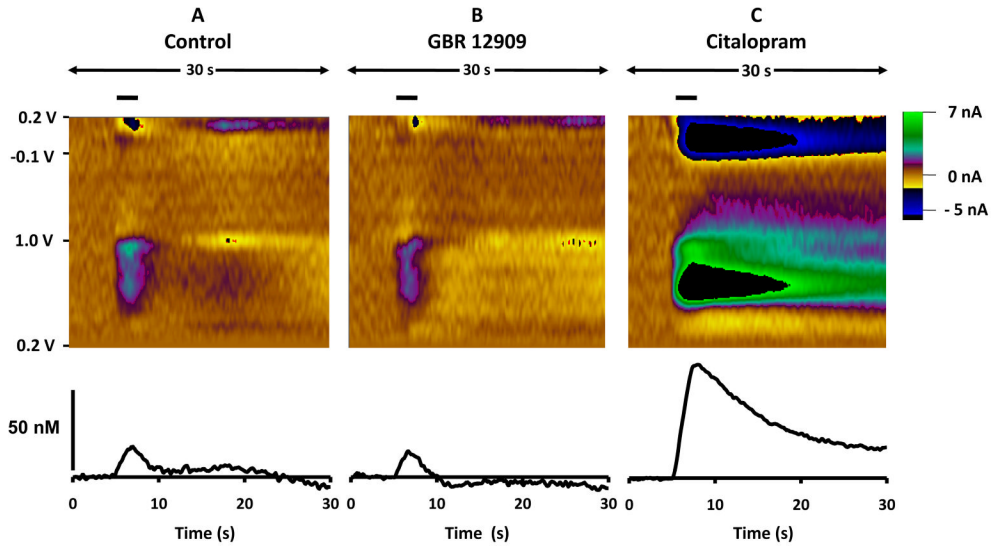

Figure 7.

Validation of 5-HT signal with GBR 12909 (15 mg kg^{-1}) and citalopram (10 mg kg^{-1}). The top panel shows the potential on y-axis plotted against time on the x-axis, the current response is represented in false color. The black blocks horizontal to the x-axis are the durations of the stimulations. The bottom traces show concentration vs. time. These responses are for **(A)** stimulation-evoked control response, **(B)** 40 minutes after systemic administration of GBR 12909 and **(C)** 40 minutes after systemic administration of citalopram.

Table 1

Comparison of Responses of Bare vs. Nafion®-Modified Microelectrodes to Common Brain Species

Averaged *in vitro* responses obtained from four electrodes for common electroactive brain species on bare and Nafion®-modified microelectrodes. The values are an average (n=4 ± SEM)

Species	Average Response		
	Bare Electrode (nA ± SEM)	Nafion Electrode (nA ± SEM)	Nafion/Bare
5-HT (1 μM)	20.8 ± 1.83	49.5 ± 10.2	2.38
Dopamine (10 μM)	8.50 ± 1.33	12.1 ± 2.61	1.42
Norepinephrine (10 μM)	8.30 ± 3.50	7.45 ± 1.42	0.90
Epinephrine (10 μM)	6.63 ± 3.80	6.32 ± 0.70	0.95
Ascorbic Acid (400 μM)	50.6 ± 15.6	20.0 ± 4.73	0.40
DOPAC (20 μM)	4.90 ± 1.17	1.55 ± 0.24	0.32
5-HIAA (10 μM)	13.8 ± 1.63	2.85 ± 0.90	0.21