analytical chemistry

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Tonic Serotonin Measurements *In Vivo* Using N-Shaped Multiple Cyclic Square Wave Voltammetry

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Cite This: https://doi.org/10.1021/acs.analchem.1c02131 Read Online

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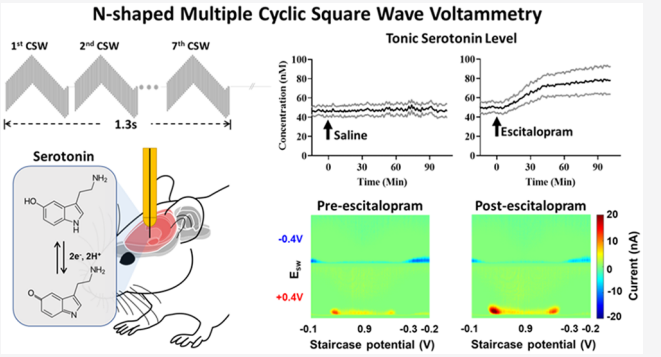
ABSTRACT: Here, we present the development of a novel voltammetric technique, N-shaped multiple cyclic square wave voltammetry (N-MCSWV) and its application *in vivo*. It allows quantitative measurements of tonic extracellular levels of serotonin *in vivo* with mitigated fouling effects. N-MCSWV enriches the electrochemical information by generating high dimensional voltammograms, which enables high sensitivity and selectivity against 5-hydroindoleacetic acid (5-HIAA), dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), histamine, ascorbic acid, norepinephrine, adenosine, and pH. Using N-MCSWV, in combination with PEDOT:Nafion-coated carbon fiber microelectrodes, a tonic serotonin concentration of 52 ± 5.8 nM (n = 20 rats, ±SEM) was determined in the substantia nigra pars reticulata of urethane-anesthetized rats. Pharmacological challenges with dopaminergic, noradrenergic, and serotonergic synaptic reuptake inhibitors supported the ability of N-MCSWV to selectively detect tonic serotonin levels *in vivo*. Overall, N-MCSWV is a novel voltammetric technique for analytical quantification of serotonin. It offers continuous monitoring of changes in tonic serotonin concentrations in the brain to further our understanding of the role of serotonin in normal behaviors and psychiatric disorders.



INTRODUCTION

Serotonin is a central neurotransmitter involved in the regulation of emotion, mood, appetite, social interactions, and sleep.[1,2] Understanding serotonergic neurotransmission in the brain is particularly important within the context of psychiatric disorders, such as forms of depression, eating and substance use disorders, post-traumatic stress disorder, and obsessive-compulsive disorder. Selective serotonin reuptake inhibitors (SSRIs) are considered first-line treatments for both depression and obsessive-compulsive disorders[1,3] and second line for eating disorders and PTSD, where cognitive behavioral therapy is generally attempted prior to pharmacological management.[4,5] Despite ongoing advances in our understanding of serotonergic signaling in psychiatric disorders, long-term pharmacological modulation of tonic serotonin levels in the brain—a core component likely mediating therapeutic responses—remains relatively unexplored. Contemporary *in vivo* recording technologies, such as microdialysis and fast-scan cyclic voltammetry (FSCV), have significant inherent drawbacks that limit their ability to investigate longitudinal changes in tonic serotonin release. As such, further advancements in our current state of knowledge will require development of new robust *in vivo* neurotransmitter monitoring techniques.

Microdialysis has been used as the gold standard method for *in vivo* measurement of tonic extracellular levels of various molecules in the brain, including serotonin.[6−8] Although microdialysis is the most frequently used method for monitoring extracellular neurochemical levels, it has relatively poor temporal resolution (collects samples every 1−10 min) and spatial resolution (the diameter of the dialysis probe membrane typically exceeds 200 μm and 1−2 mm in length where insertion may result in significant tissue disruption).[9−15] As a supplement to microdialysis, the voltammetric technique FSCV has been used for decades to detect phasic (brief bouts of endogenously or exogenously induced) changes in concentrations of electroactive neurochemical substances, such as serotonin, dopamine, norepinephrine, and adenosine.[16−21] The FSCV probe is typically a carbon fiber microelectrode (CFM) with relatively small dimensions (7 μm in diameter and 50−150 μm in length), compared to

Received: May 20, 2021
Accepted: November 18, 2021

microdialysis probes. Thus, the CFM can provide a micrometer spatial resolution of detection without incurring significant tissue damage. FSCV features a sub-second temporal response with excellent limits of detection (<5 nM for serotonin).[22−25] In addition, it has been applied to a number of animal-based studies, revolutionizing our understanding of the healthy and dysfunctional mammalian brain.[3,26−29] However, because conventional FSCV requires background subtraction of tonic electrochemical currents, it has been strictly limited to measurement of phasic changes in electroactive neurochemicals.

Recently, in an effort to overcome the inherent limitations of microdialysis, modified voltammetric techniques based on FSCV have been developed that enable the measurement of slow changes in tonic neurotransmitter levels *in vivo*.[30−35] In the context of tonic serotonin monitoring, fast-scan controlled adsorption voltammetry (FSCAV) was developed to measure tonic serotonin changes.[33] The technique applies multiple waveforms and utilizes the adsorption properties of serotonin onto the surface of the CFM. Using voltammetric techniques to measure tonic serotonin concentrations requires the elimination or isolation of the capacitive background current from the faradaic current of the electroactive species of interest without employing the conventional background subtraction methods. Unfortunately, FSCAV has not been entirely successful in eliminating capacitive components in the background currents to allow precise quantification of tonic serotonin levels. We have recently developed a technique to measure tonic dopamine concentrations, known as multiple cyclic square wave voltammetry (M-CSWV), which enabled analytical quantification of tonic dopamine concentrations *in vivo* with a relatively high temporal resolution (10 s), high sensitivity (31 pC/μM), and low limit of detection (0.17 nM). It also possesses selectivity against common *in vivo* interferents, such as ascorbic acid and DOPAC, including changes in pH.[34] By taking advantage of the strengths within the M-CSWV technique, we herein detail a novel method that can selectively quantify tonic serotonin concentrations *in vivo*.

We have recently developed an advanced voltammetric technique to record phasic changes in serotonin levels in the brain, termed N-shaped fast cyclic square wave voltammetry (N-FCSWV).[25] This new voltammetric method significantly improved the sensitivity and selectivity of phasic serotonin monitoring compared to conventional FSCV. Here, we have developed a novel voltammetric waveform based on N-FCSWV that employs multiple scanning waveforms to optimize detection of tonic serotonin concentrations in the rat substantia nigra pars reticulata (SNpr). N-shaped multiple cyclic square wave voltammetry (N-MCSWV), with high sensitivity, selectivity, and minimized signal fouling, markedly improves our ability to characterize serotonin as a potential biochemical marker of psychiatric disorders.

MATERIALS AND METHODS

Chemicals. All chemicals (such as serotonin, dopamine, 5-HIAA, and histamine) for *in vitro* and *in vivo* experiments were purchased from Sigma-Aldrich (St. Louis, MO). Buffer ("Tris") solutions were prepared by mixing 15 mM Trizma phosphate with 3.25 mM KCl, 140 mM NaCl, 1.2 mM $CaCl_2$, 1.25 mM NaH_2PO_4, 1.2 mM $MgCl_2$, and 2.0 mM Na_2SO_4, and the pH was adjusted to 7.4.

Electrode Fabrication. AS4 carbon fibers (Hexcel Corporation, Stamford, CT, USA) were used to fabricate carbon fiber microelectrodes (CFMs) as previously described.[34] The exposed carbon fiber was cut to ∼50 μm in length under a microscope. The exposed carbon fiber was then coated with PEDOT:Nafion as described in previous studies.[34,36] Briefly, PEDOT:Nafion deposition solutions consisted of 100 μL of a stock solution of 0.04 M EDOT (Sigma-Aldrich, St. Louis, MO, USA) in acetonitrile (prepared by the addition of 43 μL of EDOT to 10 mL of acetonitrile) and 200 μL of LQ-1105 Nafion (Ion Power Inc., DE, USA) in 20 mL of acetonitrile (HPLC grade, EMD Chemicals Inc., Darmstadt, Germany). The voltage for electrodeposition was controlled using a Gamry Instruments Reference 600 potentiostat (Warminster, PA, USA) in a three-electrode configuration. A tightly coiled silver wire was used as the counter electrode, and a straight silver-silver chloride (Ag/AgCl) wire was used as the reference electrode. Deposition was performed by applying a triangle waveform from +1.5 to −0.8 V at 100 mV/s for 15 cycles with an open-circuit potential prior to waveform application. After electrodeposition, all electrodes were dried at room temperature for 12 h before use.

Data Collection and Analysis. Conventional N-shaped fast-scan cyclic voltammetry (N-FSCV) was used during *in vivo* experiments.[19] N-FSCV was applied at a scan rate of 1000 V/s, with a 0.2 V holding potential versus Ag/AgCl. The switching potential of the waveform was 1.0 V and then swept down to −0.1 V and back to the holding potential.

WINCS Harmoni with WincsWare software was used to perform N-FSCV,[37] while N-MCSWV was performed with in-house written software using MATLAB (MathWork Inc., Natick, MA, USA) operating on a base station computer to apply a waveform and collect data through a data acquisition interface (NI USB-6363, National Instruments, Austin, TX, USA). An in-house built current to voltage preamplifier was used without an analog filter circuit to preserve all current responses to each square pulse in N-MCSWV. MATLAB was used to process stored N-MCSWV data in the form of a sequence of unsigned 2-byte integers. All post-signal processings including temporal averaging, filtering, and background current simulations were applied as previously described.[34] GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA) was used to create figures and statistical analysis. For all statistical comparisons, a non-normal distribution was assumed. For multiple comparisons, the Kruskal−Wallis test was used. For comparisons between two samples, either the Mann−Whitney or Wilcoxon-signed rank tests were used.

***In Vitro* Experiments.** All the *in vitro* experiments described for waveform optimization used nitrogen-purged Tris buffer solution to eliminate auto-oxidation of the electroactive analytes. Buffer solutions were maintained at 37 °C. CFM and Ag/AgCl electrodes were positioned in a beaker. Upon each trial, serotonin was introduced to examine all waveform parameter optimizations. For linearity and selectivity experiments, serotonin (10−500 nM with 100 nM representative as a physiological tonic concentration) and electroactive neurochemicals, such as dopamine (100 nM), 5-HIAA (10 μM), ascorbic acid (200 μM), histamine (1 μM), norepinephrine (1 μM), adenosine (1 μM), DOPAC (2 μM), and pH (ΔpH ±0.2), were measured with the optimized N-MCSWV.

***In Vivo* Experiments.** Adult male Sprague−Dawley rats (n = 5, for each drug group, 250−350 g) were housed in 12 h light/dark cycles and were offered food and water ad libitum.

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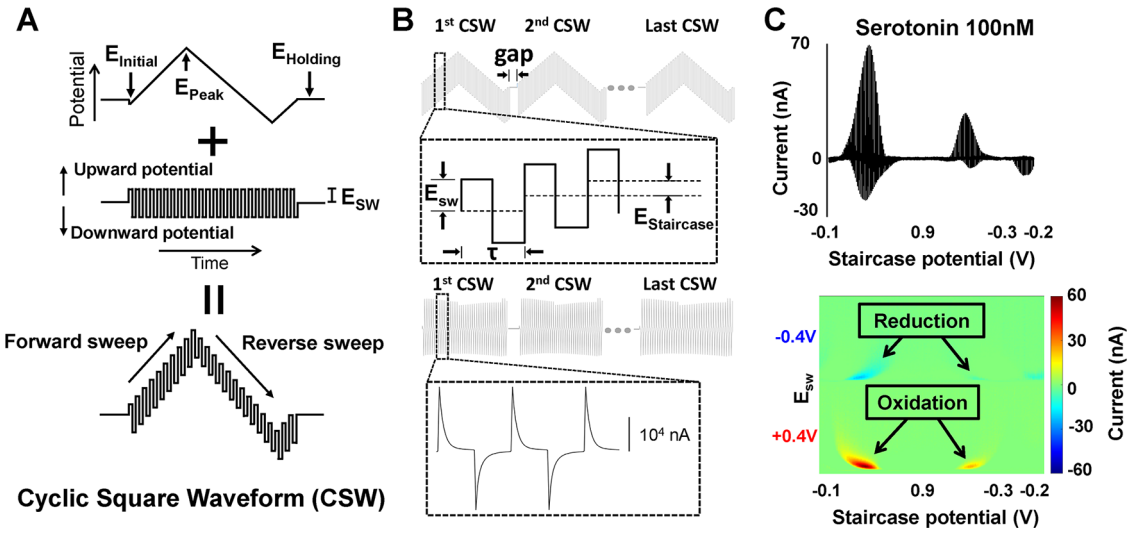

Figure 1. N-shaped multiple cyclic square wave voltammetry. (A) Schematic design of the N-shaped cyclic square waveform. (B) Design of N-MCSWV (top) and the oxidation−reduction current responses (bottom). (C) Dynamic background subtraction performed within the series of N-shaped CSWs along with capacitive background current simulation. Representative data showing a voltammogram (top) and its 2D color plot (bottom) for 100 nM serotonin in Tris solution and recorded with N-MCSWV at a carbon fiber microelectrode.

Animal care and procedures complied with the National Institutes of Health guidelines and experimental procedures approved by the Mayo Clinic Institutional Animal Care and Use Committee. Rats were anesthetized using urethane (1.5 g/kg i.p.), and stereotaxic surgeries were performed using a standard rodent surgical frame (David Kopf Instruments, Tujunga, CA, USA). A water heating pad (Adroit Medical Systems, HTP-1500, Loudon, TN, USA) was used to maintain a body temperature at 37 °C. Stimulating electrodes (Plastics One, MS 303/2, Roanoke, VA, USA) were placed unilaterally within the medial forebrain bundle (MFB) (coordinates in mm: AP −4.6, ML +1.3, DV −8.0 from dura). The CFMs were positioned unilaterally into the SNpr (AP −4.8, ML +2.0, DV −8.2) following previous studies.[25,38] A Ag/AgCl reference electrode was inserted into the superficial cortical tissue anterior to the stimulating electrodes. Electrical stimulation of the MFB consisted of trains of biphasic pulses (range of 60 Hz, 2 ms pulse width, and 350 μA pulse amplitude) applied via a WINCS Harmoni system.[37] The CFM position was adjusted until a robust stimulation-evoked serotonin signal was detected using N-FSCV. Immediately thereafter, a N-MCSWV waveform was applied at 0.1 Hz and recorded the electrochemical signal. Tonic serotonin levels were collected for an hour prior to i.p. injection of saline, 10 mg/kg escitalopram (ESCIT), 20 mg/kg nomifensine, or 20 mg/kg desipramine. Tonic serotonin levels were collected for 90 min following injection. All drugs were purchased from Sigma-Aldrich (St. Louis, MO) and were dissolved in 0.9% saline as their salt weight.

Histological Analysis. CFM and stimulation electrode locations were histologically confirmed in brain slices in the 15 animals. Animals were perfused through the left ventricle of the heart with 100−150 mL of saline followed by 100−125 mL of 4% paraformaldehyde to fix the brain *in situ*. After perfusion, the brain was removed, fixed in 4% paraformaldehyde overnight, cryo-protected in 30% sucrose, and then frozen at −70 °C in Tissue-Tek (Sakura Finetek USA., Inc., Torrance, CA, USA). After the tissue was frozen, 40 μm coronal sections were cut on a freezing microtome. The sections were mounted on glass slides and stained with cresyl violet. The locations of the CFM and stimulating electrodes were identified under light microscopy.

RESULTS AND DISCUSSION

N-Shaped Multiple Cyclic Square Wave Voltammetry (N-MCSWV). N-FSCV represents an earlier attempt to develop a fast-scan voltammetric method capable of detecting stimulation-evoked phasic changes in serotonin release *in vivo*. N-FSCV features a faster scan rate (1000 V/s) than conventional FSCV (400 V/s) as well as an N-shaped variation to the classical FSCV triangular waveform.[19] With N-FSCV, specific waveform parameters were developed to eliminate interference from dopamine and norepinephrine oxidation−reduction currents in the measurement of serotonin. However, as noted above, N-FSCV is limited to measuring phasic changes in serotonin concentrations because FSCV is a differential method that necessitates subtraction of background currents to quantify these changes relative to a zeroed baseline. Recently, we reported a novel voltammetric method called N-FCSWV, which has a unique waveform that applies a series of large amplitude square-shaped potentials superimposed onto an N-shaped waveform. N-FCSWV cycles through multiple oxidation−reduction reactions within the N-shaped waveform, thereby enhancing the sensitivity and selectivity of phasic serotonin measurements. When measuring serotonin, N-FCSWV demonstrated a significant 2.5-fold greater sensitivity than conventional N-FSCV.[25] Recently, we have also developed M-CSWV to quantify tonic dopamine concentrations *in vivo*.[39] M-CSWV uses a combination of dynamic background subtraction and simulation of the capacitive background current to eliminate the remaining capacitive background currents.[30] This allowed reliable and quantitative measurement of tonic dopamine concentrations.[34]

Combining the principles of modified cyclic square wave voltammetry used in M-CSWV with the N-FCSWV waveform, N-MCSWV provides unprecedented sensitivity, selectivity, longevity, and additional dimensional analysis in the voltammogram. The N-MCSWV waveform is based on large amplitude square waveforms superimposed onto an N-shaped

https://doi.org/10.1021/acs.analchem.1c02131
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waveform, as displayed in Figure 1A. N-shaped CSWs are applied repeatedly, capitalizing on the adsorption characteristics of serotonin at the surface of a CFM enabling tonic serotonin concentration measurements. Figure 1B demonstrates an example of the raw signal collected from a CFM in the presence of serotonin. Due to the adsorption properties of serotonin, the serotonin signal decreases with the application of successive CSWs. The final CSW in each waveform was subtracted from the second CSW to yield the difference that encompasses the serotonin signals of interest. This difference is directly related to the tonic concentration of serotonin in solution (Figure S1A). This is the concept behind dynamic background subtraction.[30] However, after subtraction, there are still some relatively small non-faradaic components remaining in the signal because of capacitive charging current changes among CSWs. To eliminate these residual non-faradaic components, the capacitive charging currents are calculated using a one-phase exponential decay equation ($Y = a \times e^{-bx}$)[34] (Figure S1B). The serotonin oxidation−reduction response to N-MCSWV is then subsequently calculated by subtracting the simulated capacitive charging currents from the raw CSW voltammograms (Figure S1C). Additionally, a voltammogram derived from square-shaped waveforms enables expanded dimensionality in measurement visualization, where converting the voltammogram to a 2D color plot provides greater electrochemical information beyond FSCV voltammograms (Figure 1C).

Optimization of N-MCSWV Parameters to Measure Serotonin. Waveform parameters, including the switching potential, amplitude of large up and down square waves (E_{SW}), increase in each stair step ($E_{Staircase}$), and the number of waveforms applied, were optimized to enhance the detection of serotonin (Figure 1A,B). The square waveform duration, tau (τ), was fixed at 1.0 ms as defined previously.[34] $E_{Initial}$ and $E_{Holding}$ were fixed at −0.1 and 0.2 V, respectively, the same as the N-FSCV waveform and N-FCSWV waveform because the waveform should begin below the serotonin's oxidation potential and maintain the serotonin adsorption between the waveform.[19,25,33] N-MCSWV was applied in 0.1 Hz repetition times to allow a sufficient period for serotonin to reach equilibrium between the solution and the carbon fiber surface. Figure 1B shows an example of the upward/downward currents generated by the upward/downward potentials. Waveform optimization experiments were all conducted with a solution containing 100 nM serotonin.

A broad range of repetition times (0.1−100 Hz) were previously used to generate distinct responses within multiple waveforms.[30,31,34] Since the serotonin response difference within CSWs is critical to determine its sensitivity, 10 CSWs were initially selected to study the decayed serotonin oxidation and reduction using the background subtraction method. The signal measured in buffer alone was subtracted from the signal measured in the presence of serotonin. It was found that the responses with 100 nM serotonin decayed 80% throughout 10 CSWs (Figure 2A). Statistical significances were obtained when comparing the sixth waveform with the 10th waveform generating serotonin signal ($n = 6$ electrodes, Mann−Whitney test, $p = 0.0260$). From the seventh waveform, there was no statistical difference when compared to the 10th waveform in the sequence ($n = 6$, Mann−Whitney test, waveform 7 vs 10: $p = 0.240$, 8 vs 10: $p = 0.818$, and 9 vs 10: $p > 0.999$). Therefore, seven CSWs were selected as the optimal number of CSWs to maximize sensitivity while minimizing the measurement time.

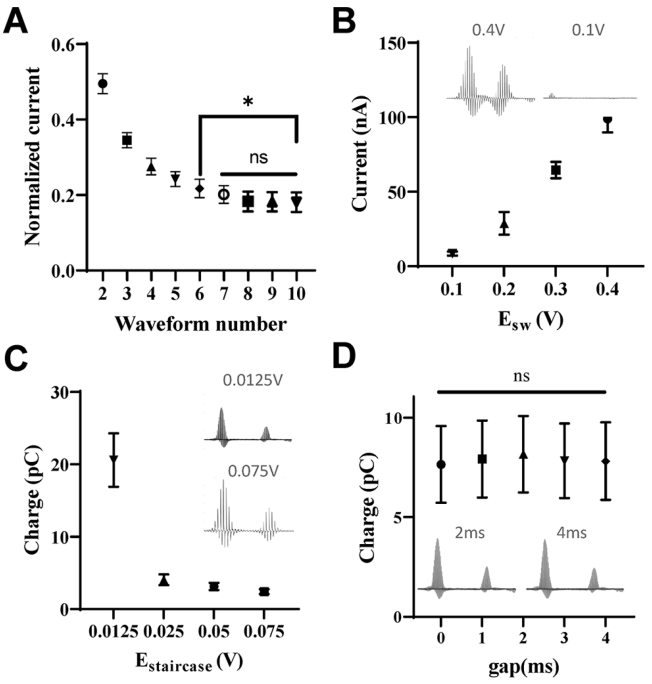

Figure 2. N-MCSWV parameter optimization. (A) Determining the number of CSWs in the single N-MCSWV waveform ($n = 6$ electrodes with ±SD, Mann−Whitney test, waveform 6 vs 10: $p = 0.0260$, 7 vs 10: $p = 0.240$, 8 vs 10: $p = 0.818$, 9 vs 10: $p > 0.999$). Optimization of the (B) E_{SW} (0.1−0.4 V), (C) $E_{Staircase}$ (0.0125−0.075 V), and (D) gap (0−4 ms) ($n = 6$ electrodes each with ±SD, non-parametric ANOVA Kruskal−Wallis test).

The amplitude of the square wave, E_{SW}, was optimized by varying the amplitude from 100 to 400 mV in 100 mV intervals. N-MCSWV response to 100 nM serotonin as the E_{SW} was varied was characterized (Figure 2B). The increases in N-MCSWV response from E_{SW} = 100 to 400 mV can be explained by the larger potential range covered for both oxidation and reduction at each individual square wave. E_{SW} was limited to 400 mV to prevent electrolysis. An E_{SW} of 400 mV was selected for N-MCSWV because of its significantly higher sensitivity than the other amplitude settings. Next, the step increment, $E_{Staircase}$, was varied from from 12.5 to 75 mV to optimize the sensitivity while preserving as many square waveforms as possible for the resolution of the 2D voltammogram. Figure 2C shows the change in the N-MCSWV response to 100 nM serotonin as $E_{Staircase}$ was varied. An $E_{Staircase}$ of 12.5 mV was chosen because it demonstrated superior sensitivity over the others. Last, the gap, the time gap of the CSWs, was examined and varied from 0 to 4 ms in 1 ms intervals. N-MCSWV response to 100 nM serotonin as the gap was varied is shown in Figure 2D. There were no statistical differences among the various gaps to the serotonin response (non-parametric analysis of variance (ANOVA) Kruskal−Wallis test). Thus, a 2.0 ms gap was chosen as an optimal time delay, which ensured a negligible voltammetric cross talk among the waveforms.[34,40,41]

Waveform Optimization to Mitigate Signal Fouling of Serotonin. Serotonin signal fouling is a well-documented phenomenon[19,42,43] and a critical impediment for tonic serotonin measurement. For both acute and chronic experiments over the course of several weeks to months, it is important to have sustained serotonin measurement with the stability. We modified various parameters of the N-MCSWV

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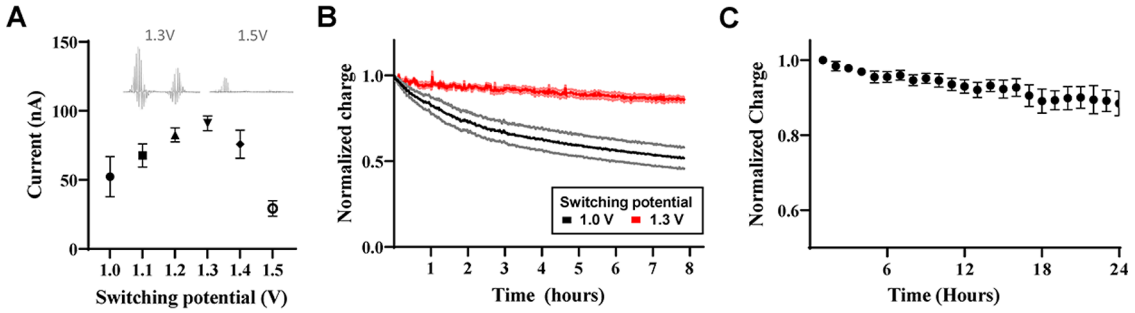

Figure 3. N-MCSWV parameter optimization for fouling mitigation. (A) Optimization switching potential (n = 6 electrodes, ±SD). (B) Comparison of the N-MCSWV waveform switching potentials of 1.0 and 1.3 V for 8 h (n = 6 electrodes). The bold line represents the normalized mean charge over time, and thin lines represent SEM. (C) Serotonin response changes in the 24 h of recording (n = 6 electrodes, ±SEM).

waveform to promote ionization of the compounds bound to the CFM surface. Through repeated modifications to the waveform, it was found that the utilization of a higher switching potential reliably mobilized adsorbed compounds, effectively cleaning the working area of the CFM and enabling continued or repeated (across multiple experiments) use.

Takmakov et al. had previously reported that a 1.3 V switching potential is sufficient to clear oxidative byproducts from the electrode surface, enabling continued measurement of electroactive neurochemicals.[44] Here, we examined the switching potential, where the upward sweep switching to the downward sweep was varied from 1.0 to 1.5 V in 100 mV intervals, and the resultant responses to 100 nM serotonin were characterized (Figure 3A). A switching potential of 1.3 V showed the highest response to serotonin. To investigate its longevity performance, the serotonin 100 nM response was monitored over 8 h *in vitro* between switching potentials of 1.0 and 1.3 V because a 1.0 V peak potential has previously been used with the conventional waveform for serotonin.[19,33] As shown in Figure 3B, a switching potential of 1.3 V reduced serotonin signal fouling more effectively than of 1.0 V, exhibiting a relatively stable serotonin response over 8 h of recording. In addition, following 24 h of applying the N-MCSWV waveform with a 1.3 V switching potential, only 12% of the original signal was lost and remained steady between the first hour and last hour (Figure 3C). A potential explanation for why this voltage enables the prolonged measurement of serotonin may include the creation or etching of new active sites on the CFM surface for adsorption.[44]

The final N-MCSWV waveform parameters for measuring tonic serotonin were E_{SW} = 0.4 V, $E_{Holding}$ = 0.2 V, $E_{Initial}$ = −0.1 V, switching potential = 1.3 V, $E_{Staircase}$ = 0.0125 V, τ = 1 ms, and the number of CSWs = 7 with 0.1 Hz repetition frequency. The duration of a single N-MCSWV scan was 1.3 s.

Sensitivity of N-MCSWV to Serotonin. To develop a method for quantifying serotonin concentrations at CFMs, the above selected parameters were utilized to obtain 2D voltammograms *in vitro* with N-MCSWV. Serotonin oxidation signals were extracted using a kernel method.[34] To construct a serotonin mask, the *in vitro* serotonin redox of N-MCSWV was used. As previously described, a thresholding method was applied in which signals greater than 60% of the maximum current were given a value of one and all remaining points were assigned a value of zero in the serotonin kernel to identify the oxidative patterns. The serotonin kernel was applied to the 2D voltammogram by element-wise multiplication, thus extracting only the serotonin feature area from the 2D voltammogram. This was then summed up to extract charge information. *In vitro*, serotonin concentrations were serially increased in time from 10 to 200 nM, and the change in charge (pC) to each concentration was recorded over time (Figure 4A). The

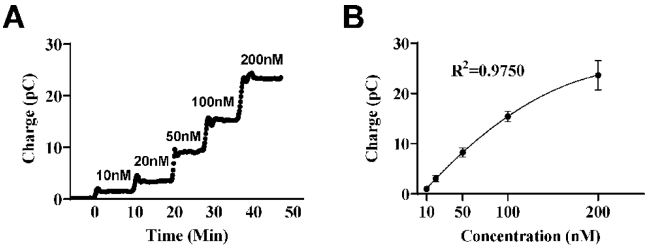

Figure 4. N-MCSWV measurement of serotonin and its sensitivity. (A) Steady-state currents in response to incremental increases in serotonin (10−200 nM) *in vitro*. (B) Concentration calibration plot of serotonin concentrations (n = 6 electrodes, ±SD, R^2 = 0.9750).

input−output steady state response of the concentration calibration curve was best modeled with a quadratic regression (Figure 4B, n = 6 ± SD, R^2 = 0.9750). The standard deviation of the concentration of 200 nM serotonin was larger than lower tested concentrations, which might be due to differences in sensitivity of the electrodes. The serotonin plot shows linearity up to 100 nM, with a sensitivity of 0.16 ± 0.01 pC nM^{-1} (n = 6 ± SEM) and a limit of detection of 0.08 nM (Figure S3).

Selectivity for Serotonin over Potential Electroactive Interferents. N-MCSWV measurement of serotonin was tested against eight common potential interferents found in the mammalian brain: the major metabolite of serotonin, 5-hydroxyindoleacetic acid (5-HIAA, 10 μM), the catecholamine neurotransmitters, dopamine (100 nM) and norepinephrine (1 μM), a primary metabolite of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC, 2 μM), a modulator of several neurotransmitters in the brain, histamine (1 μM), a major antioxidant in the brain, ascorbic acid (200 μM), the purinergic neurotransmitter, adenosine (1 μM), and pH changes (ΔpH +0.2, ΔpH −0.2). With serotonin (100 nM), N-MCSWV yielded unique oxidation and reduction patterns in the 2D voltammogram plot (Figure 5A). With 5-HIAA and other neurochemicals, their signal was significantly smaller than serotonin despite being at concentrations 10- to 100-fold higher (1 or 10 μM) than serotonin (100 nM). In comparison, 5-HIAA generated a redox current response comparable to that of serotonin with the conventional N-FSCV waveform.[19] We examined the redox response of 5-HIAA compared to the serotonin redox response to N-MCSWV. The oxidation peak

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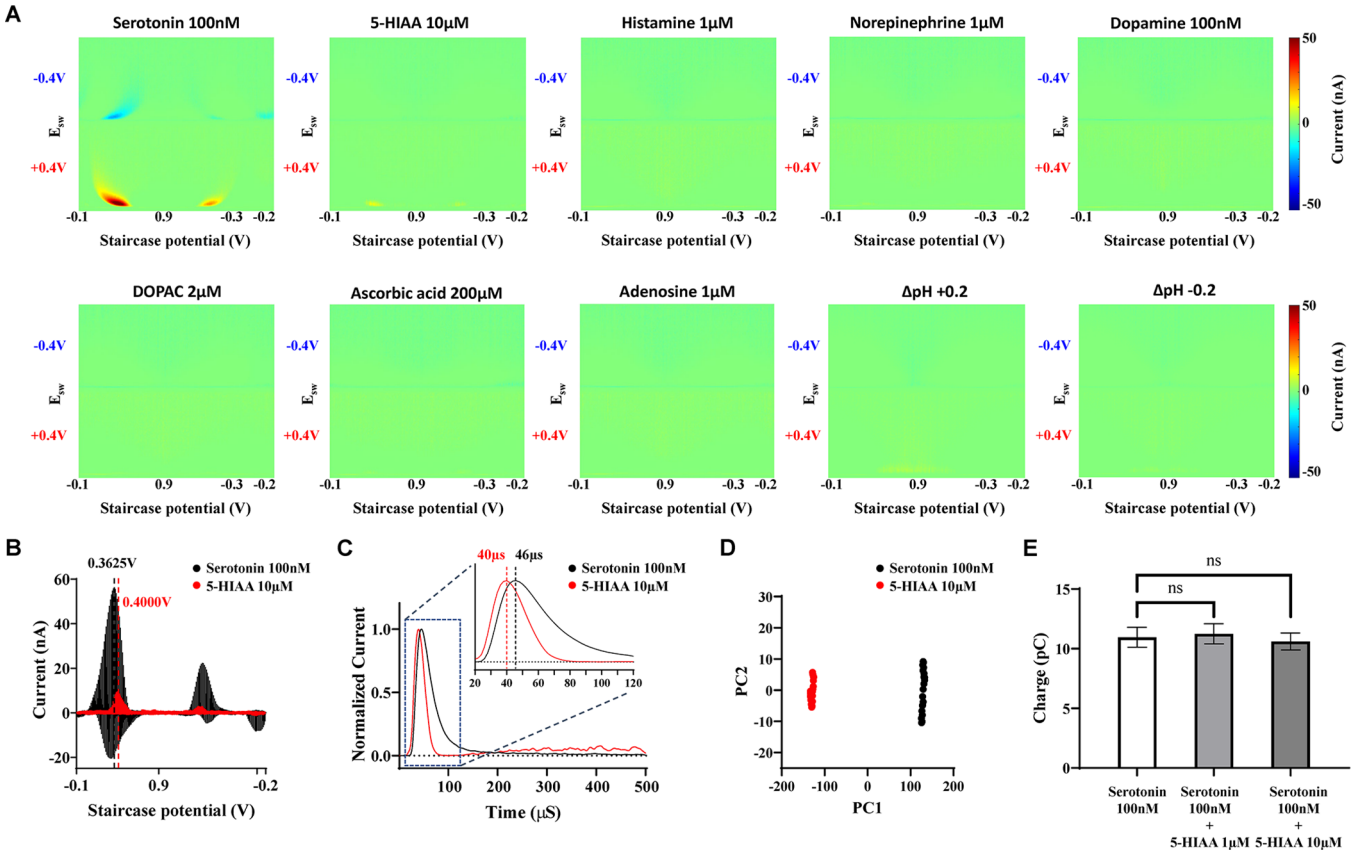

Figure 5. N-MCSWV response of various neurochemicals and its selectivity. (A) Serotonin 100 nM, 5-HIAA 10 μM, histamine 1 μM, norepinephrine 1 μM, dopamine 100 nM, DOPAC 2 μM, ascorbic acid 200 μM, adenosine 1 μM, ΔpH +0.2, and ΔpH −0.2. (B) Voltammogram response of serotonin 100 nM and 5-hydroindoleacetic acid (5-HIAA) 10 μM (right). (C) Oxidation current response changes in a single staircase of the N-MCSWV waveform. (D) Principal component analysis of the serotonin 100 nM and 5-HIAA 10 μM. (E) 5-HIAA interference to the serotonin signal, serotonin 100 nM, serotonin 100 nM + 5-HIAA 1 μM, and serotonin 100 nM + 5-HIAA 10 μM (n = 3 electrodes, non-parametric ANOVA Kruskal–Wallis test, p = 0.2458).

of serotonin was observed at a staircase potential of 0.3625 V whereas that of 5-HIAA was observed at a staircase potential of 0.4 V (Figure 5B). We also analyzed the temporal changes in the oxidation responses (Figure 5C). Serotonin oxidation reached a peak at 46 μs after the waveform was applied, and the signal decay was prolonged by approximately 100 μs. 5-HIAA oxidation reached the peak faster than that of serotonin at 40 μs, and the oxidation current decayed rapidly in 40 μs. Due to the consistency in these differences, we were able to use principal component analysis (PCA) to reliably separate the voltammograms from serotonin and 5-HIAA (Figure 5D). PCA works by transforming a variable set into a new coordinate space that maximizes variance along each of the axes. By plotting the first two PCs against each other, PCA can be seen to cluster the 5-HIAA voltammograms and the serotonin voltammograms into well-separated classes, even at 100-fold higher concentrations of 5-HIAA. Using only two principal components, a linear discriminant analysis classifier was able to achieve 100% accuracy in classifying voltammograms as coming from serotonin or from 5-HIAA across all datasets. We also examined the interference of 5-HIAA to the serotonin signal. There was no significant signal interference when comparing the serotonin signal to the serotonin and 5-HIAA mixture, each of 10- and 100-fold higher concentration of 5-HIAA present with 100 nM serotonin (Figure 5E, n = 3 electrodes, non-parametric ANOVA Kruskal–Wallis test, p = 0.2458).

Quantification of Tonic Serotonin Levels *In Vivo* Using N-MCSWV. A CFM was implanted into the substantia nigra pars reticulata (SNpr). To measure the baseline tonic level of serotonin *in vivo*, N-MCSWV was applied every 10 s to the anesthetized rats for 90 min (Figure 6). The average tonic serotonin levels were 52 ± 5.8 nM (n = 20 rats, ±SEM). These levels are 10-fold higher than those reported in previous microdialysis studies.[45−48] However, these results are highly consistent with other studies that have also utilized modified voltammetric techniques to measure tonic serotonin levels (64.9 ± 2.3 nM) in the SNpr *in vivo*.[28,33] Importantly, N-MCSWV allows superior time (a measurement every 10 s) and spatial resolution (7 μm in diameter and 50 μm in length) compared to microdialysis for *in vivo* detection of serotonin.

Above, N-MCSWV's selectivity was assessed *in vitro* with eight different neurochemicals. However, the *in vivo* environment is far more complex than can be mimicked in the beaker. Therefore, it is critical to verify the signal pharmacologically *in vivo*. Unsurprisingly, i.p. saline injection did not modify serotonin tonic levels over 105 min post-injection (Figure 6A, n = 5 rats, Wilcoxon-signed rank test, p = 0.6165). In contrast, administration of the selective serotonin reuptake inhibitor escitalopram (ESCIT, 10 mg/kg, i.p.) increased the recorded signal, reaching peak values of 45−50 min (a plateau 1.5-fold higher than pre-treatment baseline levels) after injection (Figure 6B, upper bar, n = 5 rats, Wilcoxon-signed rank test, p < 0.0001). The *in vivo* tonic serotonin selectivity

https://doi.org/10.1021/acs.analchem.1c02131
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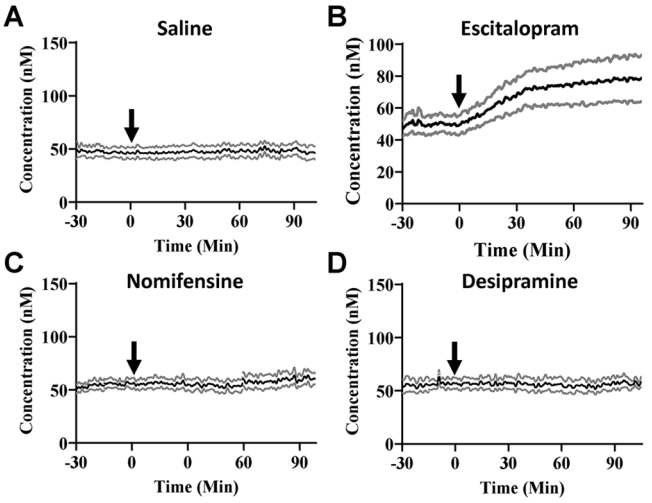

Figure 6. Pharmacological confirmation of tonic serotonin *in vivo*. Mean ± SEM tonic serotonin concentrations in response to (A) saline (n = 5), (B) escitalopram (10 mg/kg, n = 5), (C) nomifensine (20 mg/kg, n = 5), and (D) desipramine (20 mg/kg, n = 5).

was confirmed by administration of the dopamine selective reuptake inhibitor nomifensine (20 mg/kg, i.p.) and the norepinephrine reuptake inhibitor desipramine (20 mg/kg, i.p.). The responses were recorded over 105 min post-injection (n = 5 each). Each of these agents failed to significantly alter the recorded responses in the SNpr (Figure 6C,D, Wilcoxon-signed rank test, p = 0.3184, and p = 0.6165).

CONCLUSIONS

Here, we report the development and optimization of the N-MCSWV waveform (waveform length: 1.3 s, applied every 10 s) for selective detection of tonic extracellular levels of serotonin with mitigated fouling. Other approaches are significantly limited and are capable of only capturing 30 s to minute-long intervals. Our method reliably distinguished between 100 nM serotonin and 10-fold greater concentrations of eight other potential electroactive interferents. The N-MCSWV waveform was optimized with a 1.3 V switching potential to reduce signal decay over the course of 8 h of measurements *in vitro*, potentially enhancing the usable lifespan of the CFM. Finally, N-MCSWV was capable of distinguishing serotonin *in vivo* in anesthetized rats (n = 20) treated with escitalopram, with signal confirmation after challenges with a dopamine selective reuptake inhibitor, nomifensine, and a norepinephrine transporter inhibitor, desipramine. Both inhibitors were unable to alter the signal recorded by N-MCSWV, supporting the ability of this technique to selectively record tonic serotonin levels *in vivo*, paving the way for a deeper mechanistic understanding of the role of serotonin neurotransmission in both normal and psychiatric conditions.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.1c02131.

Dynamic background subtraction of N-MCSWV, sensitivity of N-MCSWV, voltammogram response of various neurochemicals, and electrode locations in rat brain slices (PDF)

Video clip showing changes in tonic serotonin levels before and after ESCIT treatment (AVI)

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Notes

The authors declare no competing financial interest.

https://doi.org/10.1021/acs.analchem.1c02131
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■ ACKNOWLEDGMENTS

This research was supported by the National Institutes of Health (NIH) 5R01NS112176-02 award and by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIT) (no. NRF-2021R1A2B5B02002437).

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