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# Improving serotonin fast-scan cyclic voltammetry detection: new waveforms to reduce electrode fouling†

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Serotonin is a neuromodulator implicated in depression that is often measured in real-time by fast-scan cyclic voltammetry (FSCV). A specialized “Jackson” waveform (JW, 0.2, 1.0 V, −0.1 V, 0.2 V, 1000 V s<sup>−1</sup>) was developed to reduce serotonin fouling, but the 1.0 V switching potential limits sensitivity and electrodes still foul. The goal of this study was to test the effects of extending the FSCV switching potential to increase serotonin sensitivity and decrease fouling. We compared the Jackson waveform, the dopamine waveform (DA, −0.4 V, 1.3 V, 400 V s<sup>−1</sup>), and two new waveforms: the extended serotonin waveform (ESW, 0.2, 1.3, −0.1, 0.2, 1000 V s<sup>−1</sup>) and extended hold serotonin waveform (EHSW, 0.2, 1.3 (hold 1 ms), −0.1, 0.2, 400 V s<sup>−1</sup>). The EHSW was the most sensitive (LOD = 0.6 nM), and the JW the least sensitive (LOD = 2.4 nM). With the Jackson waveform, electrode fouling was significant with repeated injections of serotonin or exposure to its metabolite, 5-hydroxyindoleacetic acid (5-HIAA). Using the extended waveforms, electrodes fouled 50% less than with the Jackson waveform for both analytes. No electrode fouling was observed with the dopamine waveform because of the negative holding potential. The Jackson waveform was the most selective for serotonin over dopamine (800×), and the ESW was also highly selective. All waveforms were useful for measuring serotonin with optogenetic stimulation in *Drosophila* larvae. These results provide new FSCV waveforms to measure dynamic serotonin changes with different experimental requirements, like high sensitivity (EHSW), high selectivity (ESW, JW), or eliminating electrode fouling (DA).

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## Introduction

Serotonin is a major neuromodulator in the brain that is important for mental health by regulating sleep, mood, and appetite.<sup>1,2</sup> The serotonergic system is one of the main targets of antidepressants that treat depression and anxiety disorders, but their efficacies vary in individuals.<sup>3</sup> Serotonin concentrations in the extracellular space are tightly regulated by serotonin receptors and serotonin transporters (SERT).<sup>3–5</sup> Therefore, fast analytical techniques are needed to monitor real-time serotonin changes in the brain. Electrochemical techniques are commonly used to study neurotransmitters *in vivo* in order to understand their effects on specific behaviors and

dysfunction in neurological diseases.<sup>1,2,6–8</sup> In particular, fast-scan cyclic voltammetry (FSCV) at carbon-fiber microelectrodes (CFMEs) applies linear ramp potentials at fast scan rates for high sensitivity and rapid temporal resolution detection of neurotransmitter concentration changes.<sup>1,6,9–12</sup> FSCV has revealed the dynamic co-release of serotonin and histamine in mammals<sup>13</sup> and the mechanism of selective serotonin reuptake inhibitors (SSRIs) to increase serotonin concentrations.<sup>14–16</sup> In addition, FSCV has been used to measure rapid release and uptake of serotonin in *Drosophila* larval ventral nerve cords.<sup>17–19</sup> However, serotonin remains difficult to study with FSCV because its oxidative byproducts foul CFMEs during long-term experiments.<sup>20–23</sup>

Serotonin and its major downstream metabolite, 5-hydroxyindoleacetic acid (5-HIAA), produce highly reactive radicals during oxidation that polymerize to form films on the surface of the CFME.<sup>22,23</sup> These films hinder electron transfer and cause electrode fouling, which decreases sensitivity and limits accurate measurements *in vivo*.<sup>21</sup> The standard FSCV waveform for serotonin, termed the “Jackson” waveform, was proposed to ameliorate these issues and sweeps from 0.2 V to 1.0 V to −0.1 to 0.2 V at 1000 V s<sup>−1</sup>.<sup>20</sup> The Jackson waveform was originally applied to beveled disk electrodes, however, the

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†Electronic supplementary information (ESI) available: Supplementary Fig. 1: Fast-scan cyclic voltammetry (FSCV) background (BG) charging current subtraction, Fig. 2: Precalibration and post calibration responses of electrode after *Drosophila* tissue implantation, Fig. 3: Extended serotonin waveform scan rate comparison, Fig. 4: Serotonin and 5-hydroxyindoleacetic acid oxidation scheme, Fig. 5: Fouling after long exposure to serotonin and Fig. 6: Optogenetic stimulation stability with different waveforms. See DOI: 10.1039/d0an01406k

Hashemi group showed it can also be applied to cylindrical CFMEs.<sup>21</sup> The Jackson waveform is highly selective for serotonin compared to dopamine, but electrodes still foul with repeated measurements and long exposure to 5-HIAA.<sup>21,24</sup> Surface coatings such as Nafion are commonly used to mitigate this fouling,<sup>21</sup> however they slow electrode responses.<sup>25</sup> A new FSCV waveform that prevents electrode fouling while maintaining high sensitivity and selectivity would be beneficial for studying real-time serotonin release.

Modified FSCV waveforms have been investigated for several neurotransmitters to understand how waveform parameters affect CFME sensitivity and fouling.<sup>8,24,26–28</sup> The Wightman group extended the switching potential of the dopamine waveform from 1.0 V to 1.3 V to increase sensitivity,<sup>27</sup> and later demonstrated higher switching potentials ( $\geq 1.3$  V) broke carbon–carbon bonds on the surface of the fiber,<sup>29</sup> also increasing surface oxide groups.<sup>8,11,30</sup> The higher switching potential also renews the surface to remove impurities.<sup>29</sup> Likewise, Keithley *et al.* designed “sawhorse” waveforms with an extended hold at 1.3 V and observed greater CFME sensitivity using higher scan rates ( $\geq 1000$  V s<sup>−1</sup>).<sup>26</sup> Modified sawhorse and extended switching potential waveforms also improved adenosine and histamine detection.<sup>24,31</sup> Although extending the switching potential enhanced detection of these neurotransmitters, the Jackson waveform has not been revisited in 25 years to improve serotonin detection.<sup>20</sup>

The goal of this study was to develop practical new waveforms for serotonin detection to reduce electrode fouling and increase sensitivity. We hypothesized that extending the switching potential would decrease fouling by renewing the CFME surface, and that holding at a higher switching potential would enhance these effects.<sup>29</sup> We designed new serotonin waveforms to extend the Jackson waveform to 1.3 V with varied scan rates, and tested sawhorse waveforms to hold at 1.3 V. The traditional dopamine waveform was also tested. Electrodes fouled the most using the Jackson waveform with repeated serotonin measurements and long exposure to 5-HIAA, while electrodes using the dopamine waveform did not foul. The extended waveforms with 1.3 V switching potentials had decreased electrode fouling compared to the Jackson waveform and had the highest electrode sensitivity. Waveforms were characterized *in vitro* with optogenetic stimulation in fruit fly larvae and all were useful for stable serotonin detection. Overall, our study develops extended waveforms for serotonin detection that provide high sensitivity and low electrode fouling for measurements *in vivo*.

## Experimental

### Chemicals

Serotonin hydrochloride was purchased from Sigma Aldrich (St Louis, MO). Dopamine hydrochloride and 5-hydroxyindoleacetic acid were purchased from Acros Organics (Morris Plains, NH). A 1 mM stock solution of each chemical was prepared in 0.1 M HClO<sub>4</sub>. Final working solutions were prepared

by diluting a stock in phosphate buffered saline (PBS) (131.25 mM NaCl, 3.00 mM KCl, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgCl<sub>2</sub>, 2.0 mM Na<sub>2</sub>SO<sub>4</sub>, and 1.2 mM CaCl<sub>2</sub> with the final pH adjusted to 7.4 with 1 M NaOH).

### Microelectrode preparation

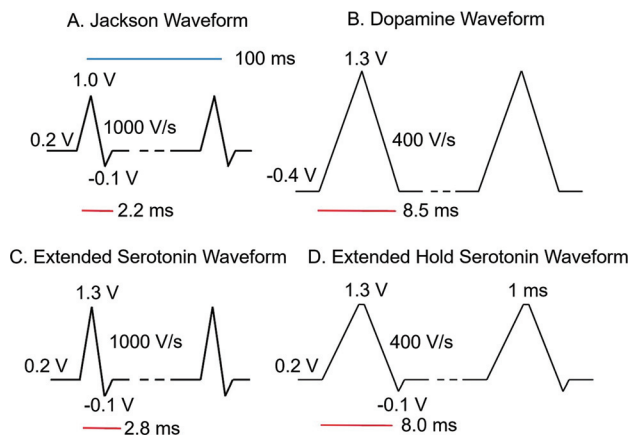
CFMEs were prepared as previously described.<sup>24</sup> A T-650 carbon fiber (Cytec Engineering Materials, West Patterson, NJ) with 7  $\mu$ m diameter was aspirated into a standard 1.28 mm inner diameter  $\times$  0.68 mm outer diameter glass capillary tube (A-M Systems, Sequim, WA) with a vacuum pump. A capillary was then pulled by a vertical puller (Narishige, Tokyo, Japan) to make two electrodes. The exposed fiber was cut to 25–75  $\mu$ m. The CFME was epoxied by dipping the tip of the fiber into a solution of 14% *m*-phenylenediamine hardener (Acros Organics, Morris Plains, NH) in Epon Resin 828 (Miller-Stephenson, Danbury, CT) at 80–85 °C for 30–40 seconds. The CFMEs were cured at 100 °C overnight and 150 °C for at least 8 hours.

### Electrochemical instrumentation

Initial flow cell fouling experiments were performed using a two-electrode system with a CFME working electrode backfilled with 1 M KCl.<sup>17,18,24,29</sup> All potential measurements are reported *versus* a chloridized Ag/AgCl wire reference electrode, and experiments were performed in a grounded Faraday cage. Before experiments, electrode tips were soaked in isopropyl alcohol for a minimum of 10 minutes to clean the surface. Electrodes were connected to a ChemClamp potentiostat and headstage (Dagan, Minneapolis, MN). Data were collected with HDCV Analysis software (Department of Chemistry, University of North Carolina at Chapel Hill). ESI Fig. 1† shows background charging currents for each waveform tested and describes background subtraction procedures. The flow-injection system consists of a six-port loop injector with an air actuator (Valco Instruments, Houston, TX). PBS buffer and test solutions were flowed at 2 mL min<sup>−1</sup> using a syringe pump (Harvard Apparatus, Holliston, MA) through a flow cell with the CFME tip inserted in solution. Analyte was flowed by the electrode for 5 seconds. *D. melanogaster in vitro* experiments were performed using the same two-electrode system, except electrodes were connected to a WaveNeuro system (Pine Research, Durham, NC). CFMEs were precalibrated and post calibrated *in vitro* using a flow injection analysis to flow 1  $\mu$ M serotonin solution by the electrode to determine the current response (Fig. S2†). The concentration of serotonin was determined *in vitro* using this calibration factor, since the measured oxidation peak current is linear with the serotonin concentration.<sup>32</sup>

### Waveform parameters

The traditional serotonin “Jackson” waveform (JW) proposed in Jackson *et al.*, scans from 0.2 V to a switching potential of 1.0 V to −0.1 V back to the holding potential of 0.2 V at 1000 V s<sup>−1</sup> (Fig. 1).<sup>20</sup> The traditional dopamine waveform (DA) was tested that scans from −0.4 V to 1.3 V at 400 V s<sup>−1</sup>. The



**Fig. 1** Waveforms tested. A. Traditional serotonin “Jackson” waveform with a 1.0 V switching potential and 1000 V s<sup>-1</sup> scan rate. B. Traditional dopamine waveform with a -0.4 V holding potential, extended 1.3 V switching potential, and 400 V s<sup>-1</sup> scan rate. C. Extended serotonin waveform (ESW) with 1.3 V switching potential and 1000 V s<sup>-1</sup> scan rate. D. Extended hold serotonin waveform (EHSW) with a 1 ms hold at 1.3 V and 400 V s<sup>-1</sup> scan rate. All waveforms were repeated at 10 Hz.

extended serotonin waveform (ESW) extends the Jackson waveform to a switching potential at 1.3 V (0.2 V, 1.3 V, -0.1 V, 0.2 V, 1000 V s<sup>-1</sup>). A sawhorse waveform, known as the extended hold serotonin waveform (EHSW) was similar to the ESW but the switching potential was held for 1 ms at 1.3 V (0.2 V, 1.3 V (1 ms), -0.1 V, 0.2 V, 400 V s<sup>-1</sup>). A frequency of 10 Hz was used for all waveforms. A 2 kHz low-pass filter was applied for 400 V s<sup>-1</sup> scan rates, and 10 kHz filter for 1000 V s<sup>-1</sup> scan rates.

### *Drosophila melanogaster* experiments

Methods were previously described in Privman *et al.* 2015.<sup>32</sup> Virgin females with UAS-CsChrimson (Stockline #55136, Bloomington *Drosophila* Stock Center, Bloomington, IN) were crossed with tph-Gal4 (Serotonin driver line), a gift from Dr Jay Hirsh (University of Virginia, Biology Department) and resulting heterozygous larvae were shielded from light and raised on standard food mixed 250:1 with 100 mM all-*trans* retinal (Sigma-Aldrich). The ventral nerve cords (VNCs) of third instar “wandering” larvae were dissected out in PBS buffer kept on ice. A VNC was placed on an uncoated Petri dish dorsal side down, and a small slice of the lateral optic lobe was removed using the tip of a 22 gauge hypodermic needle. The electrode was implanted from the lateral edge of the tissue into the dorsal medial protocerebrum. Dissection and electrode insertion were conducted under low light conditions. The electrode was allowed to equilibrate in the tissue for 10 minutes in the dark prior to data collection.

Optogenetic release of serotonin was stimulated by activating CsChrimson ion channels with red light from a 617 nm fiber-coupled high-power LED with a 200 μm core optical cable (ThorLabs, Newton, NJ, USA).<sup>32</sup> The fiber was centered above the VNC using a micromanipulator and the light was modulated with transistor-transistor logic (TTL) inputs to a T-cube LED controller (ThorLabs), which was connected to the FSCV

breakout box. TTL input was driven by electrical pulses controlled by the WaveNeuro system and HDCV software, which were used to control frequency, pulse width, and number of pulses. For *in vitro* experiments, 120 biphasic pulses were delivered at 60 Hz and pulse width of 4 ms. Stimulations were repeated every 5 minutes to allow the releasable pool of serotonin to replenish itself.<sup>17</sup>

### Serotonin imaging in larvae ventral nerve cords

*Drosophila* were bred to yield a tph-Gal4/CyO; UAS-mCD8-GFP cross, a gift from Dr Jeffery Copeland (Eastern Mennonite University, Biology Department). Third instar larvae were collected and dissected as stated above but raised in normal light conditions.<sup>32</sup> For imaging preparation, several VNCs were collected and placed in a Petri dish with cold PBS on ice. VNCs were preserved by removing PBS and pipetting 2–3 mL of 4% paraformaldehyde in PBS solution (Alfa Aesar, Ward Hill, MA). The Petri dishes were covered in Parafilm (Bemis, Neenah, WI) and gently rocked for 20 minutes on a Nutating Mixer (VWR International, Radnor, PA). Paraformaldehyde was removed and 2 mL PBS was applied for 20 minutes as an initial wash, followed by two 5-minute wash steps. A glass slide was prepared by placing preserved specimens dorsal side up in a 60 μL aliquot of Vectashield (Vector Laboratories, Burlingame, CA). A Zeiss AxioZoom microscope (Carl Zeiss Microscopy, Germany) was used to image GFP expression with Image J software (National Institutes of Health).

### Statistics

Data are the mean ± the standard error of the mean (SEM) for *n* number of electrodes. Statistics were performed in GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA). For one-way ANOVA, two-way ANOVA, and Tukey’s *post-hoc* test, significance was determined at 95% confidence level.

For sensitivity and selectivity determination, the limit of detection (LOD) for serotonin and dopamine were calculated from the lowest concentrations tested: 100 nM serotonin (all waveforms), 100 nM dopamine (DA and EHSW), 1 μM dopamine (ESW), and 10 μM dopamine (JW). LOD is calculated by a ratio method, as the ratio of the measured S/N to the tested concentration is equal to the LOD divided by 3. Noise was determined by calculating the standard deviation (SD) of the baseline current from 0–3 s in the *i vs. t* trace (*n* = 30).

## Results and discussion

### Waveform characteristics

We designed and tested new serotonin waveforms by varying the switching potential, holding potential, and scan rate to determine their sensitivity, selectivity, and electrode fouling behaviors. Fig. 1 shows the main waveforms tested and Table 1 summarizes waveform parameters. The Jackson waveform was compared to the traditional dopamine waveform that uses a negative holding potential and extended switching potential. The extended serotonin waveform (ESW) extends

**Table 1** Summary of waveform parameters

Waveform	Switching potential (V)	Holding potential (V)	Scan rate ( $\text{V s}^{-1}$ )
Jackson	1.0	0.2	1000
Dopamine	1.3	-0.4	400
ESW	1.3	0.2	1000
EHSW	1.3 (1 ms)	0.2	400

the Jackson waveform to 1.3 V, but uses the same  $1000 \text{ V s}^{-1}$  scan rate. The extended hold serotonin waveform (EHSW) is a sawhorse waveform that extends the applied switching potential at 1.3 V for 1 ms with a slower scan rate at  $400 \text{ V s}^{-1}$ . A 1 ms hold was chosen because holds  $\geq 1 \text{ ms}$  do not produce higher or different current responses, but a 1 ms hold oxidizes the surface more than a 0.5 ms hold.<sup>31</sup> Our hypothesis is that extending the switching potential will decrease fouling and increase sensitivity for serotonin by continuously regenerating the carbon fiber surface.<sup>29</sup> In addition to the main waveforms, scan rate was also varied for the ESW and EHSW (Fig. S3†).

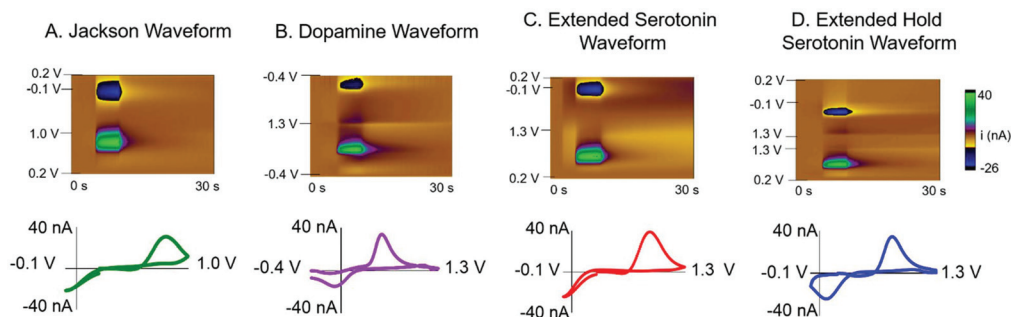
Fig. 2 shows example false color plots and cyclic voltammograms (CVs) for each waveform. The Jackson waveform (Fig. 2A) uses a high scan rate that shifts the oxidation peak to approximately 0.9 V. The characteristic reduction peak at 0.0 V is difficult to see on the CV because of the fast scan rate and potential that sweeps to only -0.1 V. However, the reduction peak is observed on the false color plot. The serotonin CV for the dopamine waveform shows full oxidation and reduction peaks at 0.6 V and 0.0 V, respectively (Fig. 2B). The serotonin CV for the ESW is similar to the Jackson waveform; however, the oxidation peak is fully observed because of the extension to 1.3 V (Fig. 2C). The cyclic voltammogram for the EHSW (Fig. 2D) is similar to the dopamine waveform and shows similar oxidation and reduction peaks. The reduction peak is easier to identify than the Jackson waveform and ESW because of the slower scan rate ( $400 \text{ V s}^{-1}$ ).

### Repeated serotonin measurement fouling

Serotonin undergoes irreversible oxidation and produces a series of radicals that dimerize and extend to form a polymer (Fig. S4†).<sup>20–23</sup> This serotonin polymer electropolymerizes to the carbon fiber and forms films that hinder electron transfer. To test electrode fouling, 25 repeated serotonin injections were made for 5 seconds every 30 seconds using flow injection analysis. A CFME fouls if the current decreases from the initial current of the first injection. Fig. 3A–D shows cyclic voltammograms for the initial (black) and 25th injections (colored) for each waveform. Electrodes using the Jackson waveform (Fig. 3A) fouled the most, with a  $39 \pm 3\%$  average current decrease after 25 injections ( $n = 6$ ). No fouling was observed with electrodes using the dopamine waveform (Fig. 3B) and current decreased only  $5 \pm 2\%$ . Electrode fouling was similar for the ESW (Fig. 3C) and EHSW (Fig. 3D) with  $19 \pm 2\%$  and  $18 \pm 4\%$  current decrease, respectively. Fig. 3E compares electrode fouling for the repeated injections among waveforms. There were significant overall effects of waveform applied (two-way ANOVA,  $F_{(3,20)} = 26.75$ ,  $p = 0.0001$ ,  $n = 6$ ) and injection number ( $F_{(24,480)} = 66.34$ ,  $p = 0.0001$ ) with significant interaction between the groups ( $F_{(72,480)} = 11.51$ ,  $p = 0.0001$ ). Tukey's *post-hoc* test revealed significant differences in electrode fouling with the Jackson waveform compared to the dopamine waveform, ESW, and EHSW (all  $p = 0.0001$ ). However, no differences in electrode fouling were observed between the other waveforms ( $p > 0.05$ ). To test the effect of scan rate on electrode fouling, the ESW and EHSW were tested at 400 and  $1000 \text{ V s}^{-1}$  (Fig. S3†). No differences were observed with scan rate for the EHSW. However, electrodes using the ESW fouled more at  $400 \text{ V s}^{-1}$  ( $39 \pm 2\%$ ) compared to  $1000 \text{ V s}^{-1}$  ( $19 \pm 2\%$ ), so  $1000 \text{ V s}^{-1}$  was chosen as the optimal scan rate.

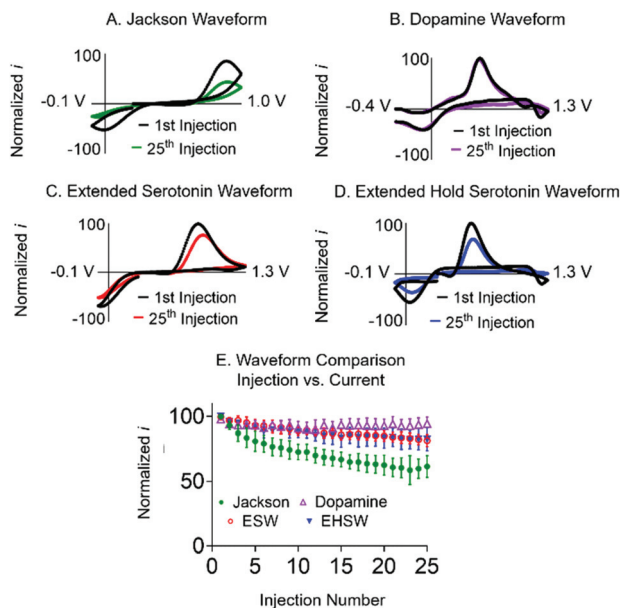
### Fouling after long exposure to 5-hydroxyindoleacetic acid

In mammals, the majority of serotonin in cerebral spinal fluid and blood quickly metabolizes to 5-hydroxyindoleacetaldehyde by monoamine oxidase-A and further oxidizes to 5-hydroxyin-

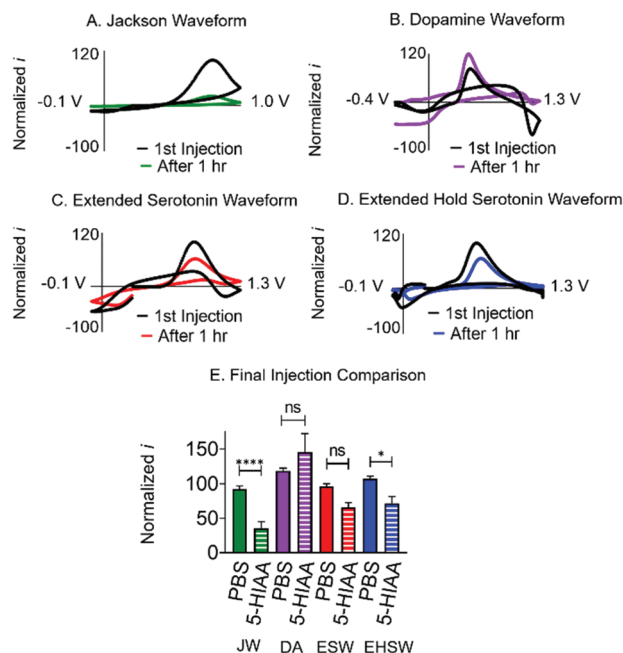


**Fig. 2** Example false color plots (above) and cyclic voltammograms (CV, below) for all waveforms for  $1 \mu\text{M}$  serotonin injection in a flow cell. Color plots show oxidation (green) and reduction (blue) for serotonin. A. The Jackson waveform displays a shifted oxidation peak in the CV, and the reduction peak is harder to see because of the -0.1 V negative potential. Both oxidation and reduction peaks are observed in the color plot. B. The dopamine waveform shows a complete CV with fully resolved oxidation and reduction peaks at approximately 0.6 V and 0.0 V, respectively. C. The extended serotonin waveform (ESW) extends the switching potential to 1.3 V with  $1000 \text{ V s}^{-1}$  scan rate and thus, the CV is similar to the Jackson waveform, except its oxidation peak is fully resolved. D. The extended hold serotonin waveform (EHSW) applies a 1 ms hold at 1.3 V ( $400 \text{ V s}^{-1}$ ) and the CV shows fully resolved oxidation and reduction peaks similar to the dopamine waveform.





**Fig. 3** Repeated serotonin measurement electrode fouling was determined by injecting  $1\ \mu\text{M}$  serotonin for 5 seconds every 30 seconds repeated 25 times in a flow cell. Cyclic voltammograms show initial (1<sup>st</sup>, black) and final (25<sup>th</sup>, color) injections for each waveform. A. The current for the 25<sup>th</sup> CV is significantly reduced with the Jackson waveform. B. Electrode current responses using the dopamine waveform were stable. Electrodes using the C. ESW and D. EHSW had slight decreases in current for the 25<sup>th</sup> injection. E. Comparison of all waveforms for fouling with repeated injections of  $1\ \mu\text{M}$  serotonin. Plot shows normalized current (to the first injection) with standard error of the mean (SEM) error bars. There were significant main effects of waveform (two-way ANOVA,  $F_{(3,20)} = 26.75$ ,  $p = 0.0001$ ,  $n = 6$ ) and injection number ( $F_{(24,480)} = 66.34$ ,  $p = 0.0001$ ) on current detected. Electrode fouling with the Jackson waveform was significantly different compared to electrodes using the dopamine waveform, ESW, and EHSW (Tukey's *post-hoc*,  $p = 0.0001$ ). No differences in fouling were observed between the other waveforms ( $p > 0.05$ ).



**Fig. 4** Electrode fouling after long exposure to 5-HIAA. A  $1\ \mu\text{M}$  5-HIAA injection was recorded and the CFME was soaked in  $1\ \mu\text{M}$  5-HIAA for 1 hour with the waveform continuously applied. A final 5-HIAA injection was performed to determine electrode fouling. The control was soaked in PBS for 1 hour between 5-HIAA injections. A. Example data using the Jackson waveform shows substantial fouling. B. No fouling was observed with the dopamine waveform in 5-HIAA, and final current values were higher than initial currents. Electrodes using the C. ESW waveform and D. EHSW fouled moderately. E. Bar graph compares responses for electrodes soaked in 5-HIAA and PBS. There were significant overall effects of waveform (two-way ANOVA,  $F_{(3,28)} = 38.16$ ,  $p = 0.0001$ ) and soaking in 5-HIAA ( $n = 6$ ) or PBS ( $n = 3$ ) ( $F_{(1,28)} = 26.47$ ,  $p = 0.0001$ ). Electrode fouling was significantly different for the Jackson waveform ( $F_{(7,28)} = 36.82$ , Tukey's *post-hoc*,  $p = 0.0001$ ) and EHSW ( $p = 0.01$ ), but not the dopamine waveform ( $p = 0.1$ ) or ESW ( $p = 0.055$ ).

doleacetic acid (5-HIAA), which is present in mammalian tissue at concentrations 1000-fold greater than serotonin.<sup>21</sup> 5-HIAA fouls CFMEs through a similar oxidation scheme to serotonin and produces a radical intermediate that dimerizes and electropolymerizes to the CFME (Fig. S4†). To determine electrode fouling effects with long 5-HIAA exposure, the current response to a  $1\ \mu\text{M}$  5-HIAA injection was recorded, the CFME was soaked in 5-HIAA for 1 hour with a waveform applied, and then the current response to 5-HIAA was analyzed again. Control experiments were similar, but the electrode was soaked in PBS for an hour with a waveform applied. CFMEs were additionally soaked in  $1\ \mu\text{M}$  serotonin for 1 hour to compare fouling behaviors to highly concentrated serotonin for an extended period of time (Fig. S5†).

Fig. 4A–D shows cyclic voltammograms for the initial and final injections of 5-HIAA for each waveform. In Fig. 4A, electrodes using the Jackson waveform show dramatically reduced currents after the waveform is applied for 1 hour in 5-HIAA. In comparison, using the dopamine waveform, currents are higher after 1 hour of soaking in 5-HIAA (Fig. 4B). For electro-

des using the ESW (Fig. 4C) and EHSW (Fig. 4D), currents decreased around a third. Fig. 4E shows a comparison of currents 1 hour after soaking in PBS (control) or 5-HIAA with the different waveforms applied. There were significant effects of waveform (two-way ANOVA,  $F_{(3,28)} = 38.16$ ,  $p = 0.0001$ ,  $n = 6$  for 5-HIAA,  $n = 3$  for PBS) and soaking in either 5-HIAA or PBS ( $F_{(1,28)} = 26.47$ ,  $p = 0.0001$ ) on current response with significant interaction between the groups ( $F_{(3,28)} = 14.79$ ,  $p = 0.0001$ ). Electrodes using the Jackson waveform fouled the most in 5-HIAA with a  $65 \pm 4\%$  decrease, and current responses were significantly different compared to the control (Fig. 4E, Tukey's *post-hoc*,  $p = 0.0001$ ). The ESW and EHSW had similar electrode fouling, with  $34 \pm 3\%$  and  $28 \pm 4\%$  current decrease, respectively. However, due to differences in variance, only EHSW current responses are significantly different in 5-HIAA and PBS ( $p = 0.01$ ). No electrode fouling was observed with the dopamine waveform, and the final currents were higher than the initial currents ( $145 \pm 11\%$ ,  $p > 0.05$ ), which indicates that the CFME is activated by the waveform. Long exposure to  $1\ \mu\text{M}$  serotonin showed similar trends to 5-HIAA even though elec-

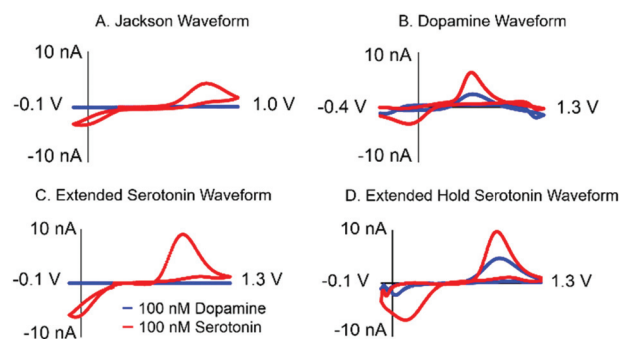
trodes fouled severely (Fig. S5†). CFMEs using the Jackson waveform fouled the most ( $85 \pm 1\%$ ) compared with the ESW ( $65 \pm 4\%$ ) and EHSW ( $63 \pm 3\%$ ), while electrodes showed remarkably less fouling with the dopamine waveform ( $22 \pm 1\%$ ). This serotonin concentration is larger than estimated basal levels ( $60 \text{ nM}$ )<sup>33</sup> so fouling *in vivo* may not be as severe.

For both serotonin and 5-HIAA fouling experiments, extending the switching potential to  $1.3 \text{ V}$  decreased electrode fouling by removing electropolymerized films.<sup>29</sup> In Jackson *et al.*, they proposed using a high scan rate of  $1000 \text{ V s}^{-1}$  to “outrun” serotonin fouling film formation.<sup>20</sup> We observed less electrode fouling using the ESW at  $1000 \text{ V s}^{-1}$  than  $400 \text{ V s}^{-1}$ , but the sawhorse waveform shows no differences with scan rate (Fig. S3†). The extended hold at  $1.3 \text{ V}$  allows more time for the carbon surface to regenerate, so fouling was similar regardless of scan rate.<sup>29</sup> Although electrode fouling was still observed with the new serotonin waveforms, fouling was half that observed using the traditional Jackson waveform for both analytes.

Using the dopamine waveform, electrodes did not foul with repeated serotonin injections or long exposure to 5-HIAA. The holding potential at  $-0.4 \text{ V}$  is applied for over 90% of a waveform cycle and helps attract the positively charged amine group to the electrode.<sup>11,27</sup> However, during serotonin oxidation, the highly reactive radical is delocalized over the indole ring structure.<sup>22,23</sup> This delocalization of the radical gives it a partial negative charge, which could reduce adsorption with a negative holding potential. Likely, serotonin dimers are still produced, but if they polymerize onto the carbon fiber, the higher switching potential regenerates the surface by continuously breaking carbon-carbon bonds.<sup>29</sup> Similarly, 5-HIAA and its radical possess a negatively charged carboxyl group at physiological pH, which is also repelled by the negative holding potential. In Fig. 4E, the dopamine waveform produced higher current responses after 1 hour because the extended switching potential increases surface oxide groups, increasing the current.<sup>29</sup> Conversely, the waveforms that use a positive holding potential at  $0.2 \text{ V}$  attract serotonin radicals and 5-HIAA onto the fiber and produce worse electrode fouling (Fig. 3E and 4E). Although extending the switching potential reduces electrode fouling, a negative holding potential is critical to eliminate it.

### Waveform sensitivity and selectivity determination

After understanding electrode fouling behaviors, we investigated CFME responses with serotonin and dopamine for each waveform to determine sensitivity and selectivity. Fig. 5 shows example cyclic voltammograms for  $100 \text{ nM}$  serotonin and dopamine with each waveform and Table 2 gives the average results. Each electrode was used to investigate current responses for both analytes, so responses could be compared ( $n = 6$ ). Electrodes using all waveforms detected  $100 \text{ nM}$  serotonin; however,  $100 \text{ nM}$  dopamine was only detected with electrodes using the dopamine waveform (Fig. 5B) and EHSW (Fig. 5D) and not with the Jackson waveform (Fig. 5A) or ESW (Fig. 5C). Current responses for serotonin were highest using



**Fig. 5** Example current responses for  $100 \text{ nM}$  serotonin and dopamine (Table 2 shows averaged results from 6 electrodes). A. When using the Jackson waveform, the electrode detected  $100 \text{ nM}$  serotonin, but not  $100 \text{ nM}$  dopamine. B. Both dopamine and serotonin were detected at  $100 \text{ nM}$  with the dopamine waveform, and the cyclic voltammograms has higher currents for serotonin. C. Using the ESW, the CFME detected  $100 \text{ nM}$  serotonin, but not  $100 \text{ nM}$  dopamine. D. With the EHSW, the electrode detected both  $100 \text{ nM}$  serotonin and dopamine, and the currents for both analytes were the highest compared to the other waveforms.

**Table 2** Serotonin and dopamine limit of detection and selectivity ratio

Waveform	Average LOD 5-HT (nM)	Average LOD DA (nM)	Ratio [DA] to [5-HT]
Jackson	$2.4 \pm 1.0$	$2000 \pm 600$	833
Dopamine	$1.5 \pm 0.3$	$9.4 \pm 0.2$	6.3
ESW	$0.8 \pm 0.2$	$189 \pm 5$	236
EHSW	$0.6 \pm 0.2$	$1.4 \pm 0.2$	2.3

$n = 6$  electrodes each.

the EHSW, followed by the ESW, dopamine waveform, and Jackson waveform.

Table 2 shows the limit of detection (LOD) for serotonin (5-HT) and dopamine (DA). The limit of detection for both analytes was calculated from the lowest concentrations detected, and the LOD for serotonin was lower than dopamine for all waveforms. CFMEs using the Jackson waveform produced the highest LOD for serotonin at  $2.4 \pm 1.0 \text{ nM}$  ( $n = 6$ ), while the LOD was lowest for the EHSW ( $0.6 \pm 0.2 \text{ nM}$ ). Electrodes using the ESW and EHSW were the most sensitive for serotonin, with LODs in the hundreds of picomolar range. Interestingly, with the traditional “dopamine” waveform, CFMEs were 6-times more sensitive for serotonin than dopamine (Fig. 5B). With the Jackson waveform, electrodes showed the greatest selectivity for serotonin, with an 800-fold higher LOD, while electrodes using ESW also had a 200-fold higher LOD for serotonin. With the dopamine waveform and EHSW, electrodes were not highly selective and produced much lower ratios (closer to 1) implying more equal sensitivity to both analytes. The LOD for dopamine with the Jackson waveform is higher than physiological concentrations typically measured *in vivo*, so electrodes should not detect it during experiments.<sup>11</sup>

Extending the switching potential increases CFME sensitivity for both serotonin and dopamine by increasing adsorption through increased oxide groups.<sup>27,29</sup> However, this decreases chemical selectivity. Electrodes using the Jackson waveform showed the greatest selectivity, followed by the ESW. In Jackson *et al.*, the  $-0.1$  V potential was designed to allow part of the reduction peak to be observed in the cyclic voltammogram.<sup>20</sup> This limited potential sweep also favors serotonin detection because it reduces around  $0.0$  V, while dopamine reduces at  $-0.2$  V. When the potential is swept only to  $-0.1$  V, the oxidized dopamine-*o*-quinone product is not recycled back to dopamine in order to be detected again on the next scan.

Interestingly, with the EHSW, electrodes did not show enhanced selectivity because of their prolonged exposure to the extended switching potential. Electrodes using this sawhorse waveform were the most sensitive to both serotonin and dopamine, however applying  $1.3$  V for  $1$  ms or longer compromises selectivity for sensitivity by increasing adsorption.<sup>27,29</sup> Keithley *et al.* examined sawhorse waveforms for dopamine detection and held at the switching potential for  $0.55$  ms.<sup>26</sup> Their LOD of  $0.9$  nM for dopamine is similar to our EHSW LOD for dopamine at  $1.4 \pm 0.2$  nM and LOD for serotonin at  $0.6 \pm 0.2$  nM.

#### Characterization of optogenetically-stimulated serotonin release using various waveforms in *Drosophila* ventral nerve cords

Previous work in the Venton lab has shown real-time serotonin and dopamine FSCV detection in larvae and adult *Drosophila melanogaster* (fruit flies).<sup>17–19,32</sup> Here, each waveform was investigated for biological applications by detecting serotonin release in isolated fruit fly larvae ventral nerve cords (VNCs). Stimulations were performed with optogenetics by inserting a genetically-encoded, light-sensitive cation channel (CsChrimson)<sup>34</sup> in cells expressing tryptophan hydroxylase (tph).<sup>32</sup> Tryptophan hydroxylase is the rate-determining enzymatic step that converts tryptophan to serotonin. CsChrimson is a form of Channelrhodopsin that responds to red light, causing exocytosis when activated. A short flash of red light onto the larval VNC causes release of only serotonin without interference from dopamine.<sup>35</sup>

Fig. 6A shows a confocal image of serotonin neurons in a 5-day old, third-instar larva. Neurons are visualized with GFP

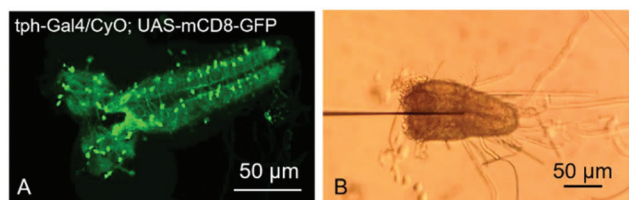
expression with a tph-Gal4/CyO; UAS-mCD8-GFP cross. Serotonin cell bodies are located on either side of the midline, although projections fill the neuropil. The CFME was inserted to the side of the midline for optimal serotonin detection and the waveform applied continuously during the experiment (Fig. 6B). The fiber optic cable was positioned above the VNC to deliver red light ( $617$  nm) stimulations, which were  $2$  seconds long and delivered every  $5$  minutes to allow the releasable pool of serotonin to replenish itself.<sup>17</sup>

Fig. 7A–D shows example false color plots, *i* vs. *t* plots, and serotonin cyclic voltammograms (inset) for the initial (colored) and final (6<sup>th</sup>, black) optogenetic stimulations for each waveform. The false color plots and cyclic voltammograms for each waveform are similar to their corresponding examples in Fig. 2A–D. The *i* vs. *t* plots show stable electrode current responses when stimulations are repeated every  $5$  minutes, regardless of the waveform used. There was no significant effect of waveform on current stability for repeated injections in *Drosophila* (Fig. S6,† one-way ANOVA,  $F_{(3,20)} = 1.747$ ,  $p = 0.1897$ ,  $n = 4$ ). Ambient levels of serotonin did not foul electrodes and stable serotonin detection was achieved because the  $5$  minutes wait period between stimulations allows the releasable pool to replenish itself.<sup>17</sup> Monoamine oxidase-A, which catalyzes the breakdown of serotonin to 5-HIAA, has not been identified in *Drosophila*.<sup>36</sup> Instead, serotonin undergoes sugar-conjugated acetylation reactions and is recycled into the larva's body and chitin in the adult's exoskeleton. Thus, fouling during *in vitro* detection may not be as much of a prevalent problem in this model organism compared to mammals.<sup>21</sup> Future experiments should compare the waveforms in more complex *in vivo* mammalian models to determine stability where 5-HIAA fouling is more prevalent.<sup>21</sup> Although detection was stable, a comparison of pre and post calibrated electrodes show CFMEs foul  $20$ – $50\%$  due to protein adsorption onto the CFME from inserting it into the VNC tissue (Fig. S2†).<sup>37</sup>

#### Comparison of serotonin waveforms and future applications

Our results show electrodes foul severely when the Jackson waveform is used to detect repeated serotonin measurements or when 5-HIAA is present. However, the Jackson waveform is highly selective for serotonin and does not detect physiological concentrations of dopamine.<sup>11</sup> The Jackson waveform is best suited for complex *in vivo* experiments where serotonin is detected with the possible interference of dopamine; for example, in regions like the striatum where interference needs to be avoided.<sup>1</sup> With the Jackson waveform, Nafion-coated electrodes are required to mitigate serotonin and 5-HIAA fouling.<sup>21</sup> Nafion is a cation-exchange polymer that shields serotonin and 5-HIAA from electropolymerizing to the surface of the fiber. Although it reduces electrode fouling, thick Nafion layers decrease response times *in vivo*, so caution is necessary when determining kinetic information during these experiments.<sup>25</sup>

Our work also shows that changing the applied FSCV waveform reduces electrode fouling without applied polymer coatings. With the extended serotonin waveform (ESW), electrodes



**Fig. 6** Serotonin neuron imaging and CFME placement in *Drosophila* larvae ventral nerve cords (VNC). A. A tph-Gal4/CyO; UAS-mCD8-GFP cross shows GFP expression of serotonin neuron clusters in the larva VNC. B. Image shows optimal CFME placement in the neuropil of the VNC to detect the highest concentrations of serotonin.



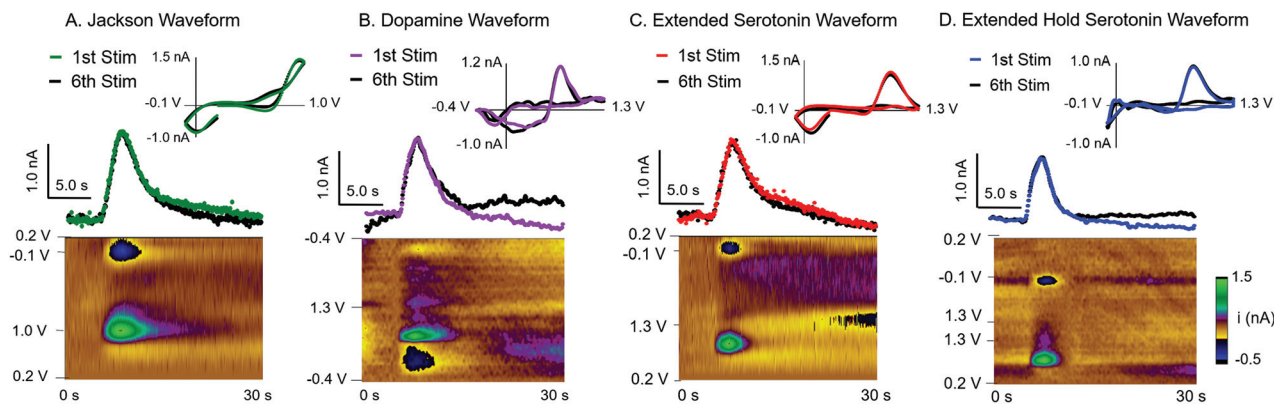


Fig. 7 Optogenetic stimulation of serotonin in *Drosophila* with different waveforms. Repeated stimulations were performed by shining a red light on the ventral nerve cord for 2 seconds every 5 minutes. False color plots show serotonin release on the first stimulation. Current responses were compared for the first and final (6<sup>th</sup>) stimulations. Electrodes using the (A) Jackson waveform, (B) dopamine waveform, (C) ESW, and (D) EHSW all produced stable measurements ( $n = 4$ ).

had comparable selectivity to the Jackson waveform with reduced fouling and higher sensitivity. The ESW could be applied *in vivo* without Nafion if selectivity and faster responses are required. Nafion coatings may not be stable with a 1.3 V switching potential because of surface regeneration.<sup>29</sup> With the extended hold serotonin waveform (EHSW), electrode responses were not as selective, but were the most sensitive for both serotonin and dopamine. The EHSW would be beneficial for optogenetic experiments where either serotonin or dopamine was specifically stimulated, especially if high sensitivity was necessary.

A major finding in this work is that there is no electrode fouling for serotonin with the dopamine waveform. Further, with the dopamine waveform, electrodes were more sensitive for serotonin than dopamine, but they were not highly selective. The dopamine waveform's anti-fouling nature is due to its extended switching potential, which renews the carbon electrode surface, and negative holding potential, which reduces adsorption of serotonin and its oxidation products. In Moran *et al.*, a unique waveform ( $-0.6$  V to  $1.4$  V and back,  $400$  V s<sup>-1</sup>) was used to detect both serotonin and dopamine in a single cyclic voltammogram, with machine learning to distinguish the compounds.<sup>1,38</sup> Serotonin and dopamine are usually identified by their different reduction peaks ( $0.0$  V and  $-0.2$  V, respectively), but these can shift *in vivo* from tissue fouling and protein adsorption.<sup>37,39</sup> While the sensitivity of CFMEs with the dopamine waveform has been investigated previously,<sup>27,29</sup> it should be recognized that the CFME is more sensitive to serotonin with this waveform and that small concentrations of serotonin will easily interfere with dopamine measurements. Ultimately, using the dopamine waveform is beneficial because it produces high electrode sensitivity with less fouling, and it is useful in experiments where selectivity is not a problem. For example, the dopamine waveform is useful for detecting serotonin in optogenetic experiments, where the channel is genetically targeted to one cell type, so selectivity is not an issue.<sup>17,32</sup>

Overall, this work shows many waveforms can be applied to CFMEs to detect serotonin. Electrodes using the Jackson waveform are the most selective for serotonin, but electrodes using the ESW show higher sensitivity than the Jackson waveform while maintaining high selectivity. With the EHSW and dopamine waveform, electrodes also have excellent sensitivity. All extended waveforms show less (ESW, EHSW) or no (DA) electrode fouling. Each waveform can be applied to *Drosophila* for stable serotonin detection; however, future *in vivo* applications of each waveform should be based on the properties desired for an experiment.

## Conclusions

Overall, this work evaluated different FSCV waveforms for serotonin detection and detailed different advantages and disadvantages for each waveform. Detection using the Jackson waveform is the most selective for serotonin but fouling is the most problematic. The ESW shows higher electrode sensitivity while maintaining high selectivity. With the EHSW and dopamine waveform, electrodes have excellent sensitivity. All extended waveforms show reduced electrode fouling compared to the Jackson waveform, and the dopamine waveform shows no electrode fouling with serotonin or 5-HIAA. Each waveform can be applied to CFMEs for stable serotonin detection in *Drosophila*; however, future *in vivo* applications should be based on experimental designs. For example, the dopamine waveform can be used in experiments where fouling is an issue if the analyte being detected is known to be serotonin, so selectivity is not a concern. The ESW is a better choice for experiments requiring selectivity between dopamine and serotonin, and will limit fouling. All waveforms should be further investigated in mammalian models, but this work developed a toolkit of serotonin waveforms that can be tuned to the requirements of an individual experiment, and will facilitate a better understanding of the role of serotonin in illnesses such as depression.



## Conflicts of interest

There are no conflicts to declare.

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