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Fast-scan cyclic voltammetry (FSCV) detection of endogenous octopamine in *Drosophila melanogaster* ventral nerve cord

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Abstract

Octopamine is an endogenous biogenic amine neurotransmitter, neurohormone, and neuromodulator in invertebrates, and has functional analogy with norepinephrine in vertebrates. Fast-scan cyclic voltammetry (FSCV) can detect rapid changes in neurotransmitters, but FSCV has not been optimized for octopamine detection *in situ*. The goal of this study was to characterize octopamine release in the ventral nerve cord of *Drosophila* larvae for the first time. An FSCV waveform was optimized so that the potential for octopamine oxidation would not be near the switching potential where interferences can occur. Endogenous octopamine release was stimulated by genetically inserting either the ATP sensitive channel, P2X₂, or the red-light sensitive channelrhodopsin, CsChrimson, into cells expressing tyrosine decarboxylase (TDC), an octopamine synthesis enzyme. To ensure that release is due to octopamine and not the precursor tyramine, the octopamine synthesis inhibitor disulfiram was applied, and the signal decreased by 80%. Stimulated release was vesicular and a 2 s continuous light stimulation of CsChrimson evoked $0.22 \pm 0.03 \mu\text{M}$ of octopamine release in the larval VNC. Repeated stimulations were stable with 2 or 5 minutes interstimulation times. With pulsed stimulations, the release was dependent on the frequency of applied light pulse. An octopamine transporter has not been identified, and blockers of the dopamine transporter and serotonin transporter had no significant effect on the clearance time of octopamine, suggesting they do not take up octopamine. This study shows that octopamine can be monitored in *Drosophila*, facilitating future studies of how octopamine release functions in the insect brain.

Keywords

Drosophila melanogaster; octopamine; electrochemistry; voltammetry; optogenetics; neuroscience

Introduction

Octopamine and its precursor, tyramine, are biogenic amines in invertebrates with distinct roles that range from modulation of muscles to complex social behaviors.^{1, 2} Octopamine acts as a neurotransmitter by regulating intracellular cyclic AMP and Ca²⁺ in *Drosophila melanogaster*.³ In larvae, octopamine/tyramine neurons in the ventral nerve cord (VNC) control locomotion, and in adults, they control locomotion, sensitivity to cocaine, and egg laying activity.^{4, 5} Octopamine also has many neuromodulatory functions including

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regulating neuromuscular transmission in larvae, and modulating learning, memory, and conditional courtship in adults.⁶⁻⁸ When released into the hemolymph, it acts as a neurohormone to mobilize lipids and carbohydrates during energy-demanding processes.^{9, 10} Octopamine is found in trace amounts in vertebrates and as a trace amine may play a role in depression and other psychiatric disorders.¹¹⁻¹³ Octopamine in invertebrates is also analogous in function to norepinephrine in vertebrates. For example, octopamine release can be stimulated by nicotine application in *Drosophila*, similar to norepinephrine release in mammals.¹⁴

Octopamine, a phenol, is an electroactive compound, and can be detected via direct electrochemistry.¹⁵ The mechanism involves a one-electron oxidation at the phenol group that creates a radical. The radical can react with other octopamine molecules to create an electroactive polymer, and a secondary oxidation peak due to oxidation of this polymer is also observed.¹⁶ Octopamine has been detected in biological samples after separation by coupling high performance liquid chromatography or capillary electrophoresis to electrochemical detection.¹⁷⁻¹⁹ Separation methods are useful in obtaining tissue content information, but are destructive and unable to provide information on real-time dynamics of octopamine.

The main electrochemical methods for studying real-time neurotransmitter release *in vivo* are amperometry, chronoamperometry, and fast-scan cyclic voltammogram (FSCV). Majdi et al. recently measured endogenous octopamine release using optogenetic stimulation in a *Drosophila* larval neuromuscular junction preparation.²⁰ They used amperometry at 0.9 V, and estimated the number of molecules of octopamine released per vesicle. Amperometry is sensitive and has high temporal resolution, but the technique is not selective and is more difficult to use in a complex preparation. Chronoamperometry measures a ratio of oxidation to reduction currents at given potentials. Fuenzalida-Urbe et al. used chronoamperometry to detect octopamine evoked by nicotine stimulation of α -bungarotoxin-sensitive nAChRs in adult *Drosophila* brains.¹⁴ With fast-scan cyclic voltammetry (FSCV), the voltage is ramped up and back to oxidize and then reduce the molecule of interest. While a background current must be subtracted, the resulting background-subtracted cyclic voltammogram (CV) helps identify the compound detected. An FSCV waveform has been optimized for stable *in vitro* analysis of octopamine at carbon fiber microelectrodes (CFMEs).¹⁶ Real-time measurements of dopamine and serotonin have been made in *Drosophila* ventral nerve cords (VNC) using FSCV but the method has not been extended to studying octopamine dynamics.^{21, 22}

Here, we optimize an FSCV waveform and detect stimulated octopamine release in the *Drosophila* larval ventral nerve cord (VNC) for the first time. By using a red-light activated ion channel, CsChrimson, there is no background shift close to the octopamine oxidation peak due to the photoelectric effect.^{23, 24} Octopamine release was also verified by picospritzing octopamine into the tissue.²⁵ CsChrimson or P2X₂, an ATP activated channel, were expressed in neurons expressing the tyrosine decarboxylase (*Tdc2*), the enzyme used to synthesize the octopamine precursor, tyramine. Since these cells have both tyramine and octopamine, disulfiram, an inhibitor of tyramine β -hydroxylase (TBH), the enzyme that converts tyramine to octopamine, was used to demonstrate that the majority of the release is octopamine.²⁶⁻²⁸ Octopamine release was vesicular and not cleared by the dopamine

transporter (DAT) or serotonin transporter (SERT). These are the first measurements of endogenous octopamine release in the *Drosophila* larval central nervous system, which will facilitate studies of the function of octopamine signaling in an intact nervous system.

Results and discussion

Visualization of octopaminergic neurons

The UAS-GAL4 system allows targeted expression of certain proteins to specific cells or tissues.²⁹ In this study, the *GAL4* gene was expressed in cells expressing *Tdc2*, neuronal tyrosine decarboxylase (TDC).³⁰ Tyrosine decarboxylase converts tyrosine to tyramine, which is then converted to octopamine by tyramine β -hydroxylase (TBH). The transcriptional activator, GAL4, activates UAS (Upstream Activation Sequence) and transcribes genes that are under its control – in this case genes coding for GFP, CsChrimson, or P2X₂. Thus, those genes are expressed only in neuronal cells expressing TDC.

To characterize the location of octopaminergic projections, the *UAS-GFP* reporter gene was expressed under the control of *Tdc2-GAL4* driver. The cell bodies, which are unpaired median neurons, are located primarily in the middle of the abdominal section of the VNC (marked a1 – a9 in Fig. 1A) and show strong GFP expression.³¹ The projections extend from the middle out to the side through the neuropil. The expression pattern is similar to previously observed patterns except that here, GFP was not expressed as strongly in the thoracic section (t1 – t3, Fig. 1A).⁴ The electrode was therefore placed in the abdominal segment 2-4 in the neuropil, where high levels of octopaminergic projections are present.

Optimization of FSCV waveform for octopamine detection

FSCV can be used to detect octopamine but an optimized waveform for *in vivo* use had not been developed. In this first study, we characterized octopamine detection with different waveforms *in situ* to optimize octopamine detection. First, a positive waveform, that held at a positive voltage, 0.1 V, scanned up to 1.3 V and back at 600 V/s was tested, because it had been optimized previously *in vitro*.¹⁶ This waveform was termed the positive waveform because of the positive holding potential. Figure 2A shows CsChrimson stimulated octopamine *in situ* (2 s stimulation) with the applied positive triangular waveform (top), cyclic voltammogram profile (middle), and color plot (bottom). The CV for the positive waveform has a peak around 1.2 V and the color plot shows that the oxidation peak is on the cathodic, downward scan very close to the switching potential. The peak potential is more shifted than for *in vitro* measurements, where the primary peak is observed around 0.9 V at this waveform,¹⁶ because the tissue can foul the electrode and slow electron transfer, changing the shape of the cyclic voltammogram.³² The positive waveform CV has very little secondary peak, because the oxidation products of octopamine are positively charged and are repelled by the positive holding potential.

Next, we tested a waveform commonly used for dopamine detection, scanning from -0.4 to 1.4 V and back at 400 V/s. The primary peak for octopamine is observed around 1.3 V, close to switching potential *in situ* (Fig. 2B). The secondary peak is around 0.7 V and the color plot shows it lasts a long time, which suggests the oxidation products of octopamine are

sticking to the electrode. When repeated calibration measurements are made *in vitro*, the response for octopamine decreases with the dopamine waveform due to fouling of the electrode by the secondary products.¹⁶

In order to move the octopamine oxidation peak away from the switching potential, we optimized a waveform using a slower scan rate than the dopamine waveform. Fig. 2C shows that with a 100 V/s scan rate, the main peak for octopamine is at 1.1 V on the anodic scan *in situ*, away from the switching potential. The secondary peak is present at 0.56 V, which aids identification, but it is not as intense and does not last as long as for the dopamine waveform. Slower scan rates do give lower Faradaic currents, as current is proportional to scan rate. Decreasing the scan rate does decrease the sensitivity from an average of 12 ± 1 nA/ μ M for 400 V/s to 4.3 ± 0.5 nA/ μ M for 100 V/s. However, noise is also proportional to background current and decreases as well. The average S/N ratio for octopamine detection for 1 μ M octopamine with the slower scan waveform is 154, which is 88% of the average S/N ratio of 174 for the dopamine waveform. The LOD calculated from the average S/N ratios of 200 nM octopamine for the dopamine waveform is 14 nM and for the slower scan waveform it is 19 nM.³³ The slower scan waveform was chosen for the rest of the studies because the background current is most unstable at the switching potential, and so errors (such as pressure changes with pressure ejection) often occur at this potential. Errors at the switching potential are a problem when using blue light sensitive Channelrhodopsin (ChR2) to mediate octopamine release because the photoelectric effect causes small currents at the switching potential.²⁴ Thus, using the slower scan rate waveform keeps the main peak away from the switching potential and allows the secondary peak to be used as an identifier. While the secondary peak is present for this waveform, we show *vide infra* that stable current responses are observed for octopamine upon repeated stimulations.

Confirmation the released compound is octopamine and that release is vesicular

Since the CsChrimson is expressed in cells expressing the tyramine synthesis enzyme, TDC, they could be releasing octopamine or tyramine. Disulfiram, a tyramine β -hydroxylase (TBH) inhibitor, was applied to block the synthesis of octopamine from tyramine. Thus, a decrease in stimulated release after disulfiram would confirm that the release was octopamine.²⁶⁻²⁸ Continuous, 2 s red light stimulations of CsChrimson were repeated every 5 min after bathing VNCs in 100 μ M disulfiram for 15 min. Results were normalized to the value of the first stimulation. For control flies, release from repeated stimulations were consistent and the eighth stimulation (45 mins in buffer + DMSO) was $94 \pm 3\%$ of the first, which was not a significant decrease (One-way ANOVA, Bonferroni post-test, $p > 0.05$) (Figure 3). Disulfiram significantly decreased evoked current (one-way ANOVA, $p < 0.0001$, $n = 6$), and the current decreased to $19 \pm 3\%$ after the eighth stimulation (45 mins in disulfiram) (Bonferroni post-test, $p < 0.05$). The magnitude of the decrease is similar to the decrease in stimulated serotonin after the use of a serotonin synthesis inhibitor.³⁴ This substantial decrease in current after disulfiram indicates that the majority of the evoked species is octopamine. Future studies could also confirm this genetically by blocking targeted expression of the enzyme tyramine β -hydroxylase with RNAi. However, a stable homozygous line expressing *tdc2-GAL4; UAS-CsChrimson* is not currently available, so all

three genes – *tdc2-GAL4*, *UAS-CsChrimson*, and *UAS-RNAi^{TβH}* – could not be expressed together.

Neurotransmitters are contained in vesicles, which release their contents into the synaptic cleft by exocytosis after an action potential. To determine if octopamine release is vesicular, VNCs were bathed in 100 μM reserpine, a vesicular monoamine transporter (VMAT) inhibitor. VMAT is a protein that transports neurotransmitters from the cytosol into synaptic vesicles for storage and subsequent release. There was a significant effect of reserpine on evoked current (one-way ANOVA, $p < 0.0001$, $n = 6$) and evoked current decreased $33 \pm 7\%$ on the eighth stimulation (45 mins in reserpine) (Bonferroni post-test, $p < 0.05$ compared to control). Reserpine is likely not blocking 100% of VMAT or the releasable pool may not be depleted, so the response does not go to zero. The results confirm that optogenetic stimulation is causing vesicular release of octopamine. The vesicular nature of octopamine release could also be probed further with genetic manipulation of the VMAT gene in the future.³⁵

Comparison of puffed on, CsChrimson, and P2X₂-mediated octopamine release

The CVs and color plot profiles of stimulated release were compared to those obtained from puffing octopamine into VNCs. Octopamine (0.01 pmol) was applied in the VNC using a picospritzing pipette (Figure 4A). Stimulated release was compared for optically activated CsChrimson channels and ATP activated P2X₂ channels. Figure 4B shows the CV (top right), concentration vs. time (center), and color plot (bottom) obtained for 2 s continuous red light stimulation of *Tdc2-GAL4; UAS-CsChrimson* larval VNC. On average, a 2 s stimulation of CsChrimson released 0.22 ± 0.03 μM octopamine. Figure 4C shows similar plots for ATP stimulation of *Tdc2-GAL4; UAS-P2X₂* larval VNC preparation. On average, ATP stimulation released 0.33 ± 0.06 μM of octopamine. The higher level of release with ATP compared to 2 s red light stimulation is consistent with previous observations of stimulated dopamine release.^{24,36} The CVs for octopamine stimulated by CsChrimson and P2X₂ are similar to the CV for exogenously applied octopamine with primary peaks around 1.1 V and secondary peaks around 0.5 V. The peak close to 1.3 V on the CV for ATP stimulated release is an artifact (as marked in Figure 4C), which is likely due to background shifts from pressure ejection or ATP oxidation.³⁶ With a higher scan rate waveform, this artifact would interfere with the primary oxidation peak for octopamine, but at 100 V/s, the octopamine oxidation peak and the artifact are well separated and there is no interference. The concentration vs. time plot for CsChrimson stimulated release has a sharper peak than ATP stimulated release, because the timing is easier to control with light stimulations than with ATP, which must diffuse through the tissue and is present longer. This longer course of ATP stimulation also explains the higher levels of release and longer lasting secondary peaks obtained compared to optogenetic stimulation. In future experiments, either light or ATP-mediated release could be used to study octopamine dynamics.

Stability of release and effect of stimulation frequency

Red light-stimulated release via CsChrimson was further characterized by studying the stability of octopamine release and the effect of interstimulation times on stability. Two second duration, continuous red light stimulations were repeated every 2 or 5 minutes. The

release was stable with both 2 minute (one-way ANOVA, $p = 0.2651$, $n = 7$) and 5 minute (one-way ANOVA, $p = 0.8197$, $n = 7$) interstimulation times (Figure 5A). Similarly, a stable current response was also obtained upon repeated exposures to octopamine at these intervals for *in vitro* calibrations (one-way ANOVA, $p > 0.5$, $n = 4$) (Fig. 5B). Stable release suggests that the secondary oxidation product does not foul the electrode and cause changes in sensitivity on this time scale. The standard error of the mean was higher and currents slightly lower for the 2 minute interstimulation time; hence a 5 minute interstimulation time was used for further experiments.

Continuous red light stimulations were varied between 0.1 s and 3 s in order to test the effect of stimulation length on octopamine release (Figure 6A). Octopamine oxidation current increased with increasing duration of light stimulation up to 0.5 s. With stimulations longer than 1 s, the evoked current decreased after reaching a maximum level, even while the light was still on. This decreasing response is different than the signals for dopamine and serotonin that keep increasing or reach steady state during long stimulations.^{22, 34} The results suggest that the releasable pool for octopamine is more limited than for serotonin or dopamine in similar preparations.

Pulsed light stimulations are often used to mimic neuronal firing patterns.^{37, 38} To understand the effect of pulsed stimulation and changing frequency of stimulation, pulse trains were studied in two ways: (1) holding the stimulation time constant at 2 s (i.e. changing the frequency and number of pulses), and (2) holding the duration of the light on constant for 2 s (i.e. keeping the number of pulses constant at 500 pulses while changing the frequency). All light pulses were 4 ms in length. The currents were normalized to a 2 s continuous stimulation for each sample. For the first stimulation method with constant stimulation time, current was dependent on the frequency of pulsed light (Fig. 6B, One-way ANOVA, $p < 0.0001$, $n = 7$), and was significantly different from the 2 s continuous stimulation up to 60 Hz (Bonferroni's post-test, $p < 0.05$). Figure 6B also shows example current vs. time traces that illustrate that higher concentrations of octopamine were released with higher frequencies. For the second stimulation method, with constant pulses, there was a significant main effect of frequency (Fig. 6C, one-way ANOVA, $p < 0.0001$, $n = 7$) and post-tests showed that signals were significantly different than the 2 s continuous stimulation up to 80 Hz (Bonferroni's post-test, $p < 0.05$). The concentration vs. time plots (Figure 6C) show that the concentration of octopamine decreases with time as the length of stimulation increases, which is clearly seen in the 20 Hz plot. Similar to the continuous stimulations (Fig. 6A), the concentration of octopamine decreases during the stimulation, which implies that the releasable pool can be easily depleted. In contrast, for other neurotransmitters like serotonin, a steady state response is observed at lower frequencies instead of a decrease.³⁸ Overall, results of the pulsed stimulation tests show the evoked current is dependent on the frequency of light; however the octopamine release is depleted with longer stimulations and decreases while the light stimulation is being applied.

Characterization of octopamine clearance

Most monoamine neurotransmitters are cleared back into cells via a transporter, but an octopamine transporter has not been identified in *Drosophila*.³⁹ Octopamine has a similar

structure to dopamine, and it has been suggested that the dopamine transporter could transport octopamine.⁴⁰ However, tests of *Drosophila* dopamine transporter (dDAT) expressed in MDCK cells found that octopamine was not a good substrate for dDAT.⁴¹ *Drosophila* serotonin transporter (dSERT) expressed in HeLa cells and *Xenopus oocytes* have also shown little or no activity for octopamine.^{42, 43} However, the clearance of octopamine by known monoamine transporters has not been studied in the VNC. We tested the extent to which the dDAT or dSERT affect octopamine clearance by using nisoxetine to inhibit dDAT and fluoxetine to inhibit dSERT.^{22, 44} Fig. 7 shows example data before and after the uptake inhibitors. There is little effect of 20 μ M nisoxetine, and 100 μ M fluoxetine increases the peak height but not the clearance time. To quantify clearance, t_{50} , the time the concentration vs. time curve to decay from the peak to 50% was calculated and it was not significantly different for nisoxetine or fluoxetine compared to control values (paired t test, $p > 0.1$, $n = 5 - 8$) (Table 1). Thus, dSERT or dDAT do not uptake octopamine.

The t_{50} value of 1.4 s for octopamine is lower than that for serotonin (~ 2.5 s).³⁸ The rate of diffusion will be similar for both neurotransmitters as they are in similar environment. The small t_{50} value, therefore, suggests that octopamine is either metabolized quickly or transported into cells by some other transporters. N-acetylation is the major metabolic pathway for neurotransmitter inactivation in *Drosophila*, but metabolism is expected to act on a slower time scale than reuptake.⁴⁵ Genome wide searches for transporter DNA have not found any other transporters for octopamine, so future research into mechanisms of clearance in *Drosophila* would be valuable.³⁹

Although the t_{50} values were not significantly different in the presence of the transporter inhibitors, evoked octopamine release was significantly higher in the presence of fluoxetine (paired t-test, $**p = 0.0078$, $n = 7$) (Table 1). This result is similar to previous observations of increased norepinephrine in the presence of fluoxetine in rat brains, and is another indication for the analogy between norepinephrine in vertebrates and octopamine in invertebrates.⁴⁴ The increase of norepinephrine in rat brains was 5-HT_{2C} receptor mediated, because fluoxetine is also a 5-HT_{2C} antagonist. However, there is no 5-HT_{2C} receptor homolog reported in *Drosophila*. Other 5-HT receptor homologs have been identified, such as Dm5-HT_{2a}, but it has pharmacological properties similar to 5-HT_{2B} receptor in mammals and has not been identified as a 5-HT_{2C} receptor homolog.^{46, 47} These results suggest that future experiments might examine the ability of serotonin receptors to mediate increases in octopamine in *Drosophila*.

Comparison of octopamine release with dopamine and serotonin

The concentration of octopamine released with 2 s red light stimulation ($0.22 \pm 0.03 \mu$ M) is slightly lower than stimulated dopamine or serotonin release in similar preparations.^{24, 34} For both serotonin and dopamine release, a 2 s continuous light stimulation produces a peak that after the stimulation ends, but for octopamine, the peak release begins to decrease during the stimulation for a 1 s continuous stimulation. These results suggest a smaller octopamine releasable pool, which was unexpected as octopamine tissue content is higher than both serotonin or dopamine in the larval VNCs.¹⁷ There are several reasons why the stimulated octopamine release could be lower than expected. First, the lower release could

mean that *Tdc2-GAL4* is not as strong a driver as that used for dopaminergic (*TH-GAL4*) and serotonergic (*TPH-GAL4*) neurons. The *Tdc2-GAL4* line used here (Bloomington fly stock 9313) was not made utilizing the site-specificity of attB and attP on DNA and could have resulted in less expression of the driver and thus CsChrimson expression would be lower.⁴⁸ Second, octopamine transporters have not been identified in *Drosophila* and the lower release could be because there is no rapid recycling of octopamine through transporters to continually replenish the releasable pool.³⁹ Finally, the release of octopamine was measured in the neuropil of the VNC, but the GFP images (Fig. 1) indicate that there may not be as much arborization and terminal as there are for dopaminergic or serotonergic terminals in that region. Future studies could examine regional differences in octopamine release both in the larval and also adult CNS of *Drosophila*.

Conclusions

We have detected and quantified stimulated endogenous octopamine release with FSCV for the first time. Optogenetic stimulation or activation of an exogenous ATP channel was used to stimulate vesicular octopamine release. Release was stable with 2 or 5 minutes interstimulation times, and dependent on the frequency of the applied light. Long stimulations cause decreasing response, so stimulations of 1 s or shorter should be used in future studies. Although the clearance of octopamine was rapid, there was no evidence of octopamine transport through dopamine or serotonin transporters suggesting an alternative method of clearance. The serotonin transporter inhibitor, fluoxetine, did increase the amount of octopamine release, suggesting that future studies are needed to investigate the effect of serotonin on octopamine release. Overall, FSCV can be used to measure octopamine release and clearance rates in larvae, providing insight into the mechanisms of action of this invertebrate neurotransmitter.

Experimental Section

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), and solutions were prepared in Milli Q water (Millipore, Billerica, MA) unless noted otherwise. Electrode calibrations were performed in phosphate buffer solution (PBS) (131.25 mM NaCl, 3.0 mM KCl, 10.0 mM NaH₂PO₄, 1.2 mM MgCl₂, 2.0 mM Na₂SO₄, and 1.2 mM CaCl₂) with pH adjusted to 7.4, which was made once a month and stored at 4°C. To make the larval dissection buffer, 11.1 mM glucose and 5.3 mM trehalose were added to the PBS buffer on the day of the experiment. For P2X₂ experiments, ATP solution (0.1 mM) was also prepared daily in the dissection buffer. Ten mM stock solutions of octopamine were prepared in 0.1 M HClO₄ once a month and stored at 4°C. Diluted octopamine solutions for electrode calibration and pressure ejection experiments were prepared daily in PBS buffer.

Disulfiram (Tocris Bioscience, Ellisville, MO) and reserpine (Sigma-Aldrich, St. Louis, MO) stock solutions of 10 mM were prepared in DMSO, and diluted to 400 μ M in PBS. Fluoxetine (400 μ M) and nisoxetine (80 μ M) were prepared in PBS. To add drug to the VNC, 1 mL of the respective solution was added to the Petri dish that contained 3 mL of dissection buffer.

Drosophila and VNC preparation

Drosophila melanogaster stocks were maintained and crossed as described before.²⁴ Males with *Tdc2-GAL4* on the second chromosome (Bloomington fly stock #9313, gift of Jay Hirsh, University of Virginia) were used to express the following UAS constructs (on the third chromosome): *UAS-CsChrimson* and *UAS-P2X₂* (gifts of Vivek Jayaraman, Janelia Farm Research Campus) by crossing *Tdc2-GAL4* males with respective virgin females. Resulting *tdc2-GAL4; UAS-CsChrimson* larvae were fed standard cornmeal food with 400 μ M all-trans-retinal and kept in the dark. Larvae were dissected in phosphate buffer and prepared using previously described procedures.²⁴ Briefly, the central nervous system was dissected out from a third instar larva using fine tweezers, the optic lobes cut off, and the VNC was transferred to the lid of a Petri dish with 3 mL of the dissection buffer in it. For ATP stimulation and octopamine pressure ejection studies, the opposite end of the VNC was cut with fine scissors to facilitate micropipette insertion as described previously.⁴⁹

Electrochemical setup, light stimulation, and data analysis

Carbon-fiber microelectrodes (CFMEs) were fabricated with 7 μ m diameter T-650 carbon fibers (Cytec Engineering Materials, West Patterson, NJ) in a 1.2 mm o.d. glass capillary (A-M systems, Carlsburg, WA) pulled to a tip. CFMEs were cut to 50 - 75 μ m to form cylindrical electrodes. Data were collected with Dagan Chem-Clamp potentiostat (Dagan, Minneapolis, MN, $n = 0.01$ headstage), PCI 6711 and 6052 computer interface cards (National Instruments, Austin, TX) and a home-built breakout box. Tar Heel CV software (gift of Mark Wightman, University of North Carolina) was used for data collection and analysis. Electrodes were precalibrated with 1 μ M octopamine in a flow cell. Electrodes were also calibrated in the presence of disulfiram and reserpine in the buffer to test any effects on sensitivity. There was no significant effect of either disulfiram or reserpine on electrode sensitivity (paired t-test, $p > 0.1$, $n = 4$).

The VNC and electrode were viewed under a 40X water immersion lens (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA) and the electrode was inserted in the neuropil from the second to forth abdominal section using a micromanipulator (Narshige N-MMN-1 and N-MMO-202ND). The potential was applied between the CFME and a Ag/AgCl reference electrode in the bath. Optical stimulation was performed with 617 nm LED (~0.2 mW) and driver (ThorLabs, Newton, NJ) with optical fiber placed ~400 μ m from the VNC.

A picospritzing pipette was made with the same glass capillary and vertical puller used to fabricate CFMEs. After being pulled, the pipette tips were trimmed and polished to a 30° angle using a microelectrode beveller (Sutter Instrument Co., Novato, CA, USA). The pipette was filled with 0.1 mM ATP and inserted into the VNC to pressure eject ATP using Picospritzer III instrument (Parker Hannifin, Fairfield, NJ). The pipette was calibrated by measuring the diameter of liquid ejected in oil at a set pressure and ejection time. A similar procedure was used for pressure ejection of 20 μ M octopamine.

All statistics were performed with GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA). Significance was measured with a 95% confidence interval. Graphs are shown as mean $\pm$ standard error of the mean.

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Figure 1.

Visualization of neurons expressing tyrosine decarboxylase (*Tdc*) in *Drosophila melanogaster* larval ventral nerve cord (VNC). (A) Bright field image of a larval VNC and (B) image of the same VNC expressing GFP in neurons with tyrosine decarboxylase (*Tdc2-GAL4; UAS-GFP*). (C) Overlay of GFP expression on the image of the VNC. Abdominal section (a1 – a9) and thoracic section (t1 – t3) are indicated on the right. Strong GFP expression (green) is seen on the unpaired median neurons in the abdominal section, with projections into the neuropil on either side.

Figure 2.

Optimization of FSCV waveform for octopamine detection. The applied waveform (top), cyclic voltammogram (center) and color plot (bottom) for octopamine detected with (A) positive waveform, (B) dopamine waveform and (C) slower scan waveform. The 2 s stimulation is marked by a red line under the color plot.

Figure 3.

Confirmation of the detection of octopamine and that the release is vesicular. After the initial stimulation pre-drug (stimulation number 1), the VNCs were bathed in 100 μ M disulfiram to inhibit octopamine synthesis, or 100 μ M reserpine, to block the vesicular monoamine transporter. After 15 minutes in drug, stimulations were performed every 5 minutes after the second stimulation. The evoked current is significantly lower than control for both disulfiram and reserpine (one-way ANOVA, $p < 0.0001$, $n = 6$).

Figure 4.

Comparison of puffed octopamine and different stimulations. Cyclic voltammograms (top), concentration vs. time (center), and color plots (bottom) for (A) octopamine puffed into a larval VNC (B) CsChrimson (red light, 2s) mediated release and (C) P2X₂ (0.5 pmol ATP) mediated release in larval VNC. All show similar peak characteristics with the primary peak around 1.1 V and secondary peak around 0.5 V with the slower scan waveform.

Figure 5. Stability of CsChrimson stimulated octopamine release. (A) The release evoked by 2 s red light stimulation is stable for 2 min inter-stimulation time (ANOVA, $p = 0.2651$, $n = 7$) and 5 min inter-stimulation time (ANOVA, $p = 0.8197$, $n = 7$). (B) Stable responses are also observed *in vitro* calibrations for 2 s octopamine injections with 2 minutes (ANOVA $p = 0.7441$, $n = 4$) or 5 minutes ($p = 0.9920$, $n = 4$) interevent times.

(A) Continuous stim. of varying lengths

(B) Total stim. time: 2s

(C) Pulse number: 500

Figure 6.

Effects of length of continuous light stimulation and frequency of pulsed stimulations. (A) Concentration vs. time plots for continuous light stimulation of different durations. Colored bars at bottom show total stimulation times. (B-C) Effect of pulsed stimulation frequency. (B) The total stimulation time was constant at 2 s and pulse length was 4 ms. Data are normalized to 2 s continuous stimulation set to 100 %. Current due to evoked octopamine is dependent on the frequency of applied light and significantly different than the 2 s continuous stimulation up to 60 Hz (one way ANOVA, $p < 0.0001$, $n = 7$). Example concentration vs. time plots are shown below with colored bars showing total stimulation time. (C) The total number of pulses was kept constant at 500 and length of pulse at 4 ms so the total time light is on is 2 seconds. There was a significant effect of frequency up to 80 Hz (Bonferroni's multiple comparisons test, $p < 0.05$).

Figure 7. Effect of uptake inhibitors. Example concentration vs. time plots for 1 s CsChrimson stimulation pre-drug (black traces) and after 15 minutes in (A) 20 μM nisoxetine, or (B) 100 μM fluoxetine (red traces).

Table 1

Effect of dopamine transporter inhibitor (nisoxetine) and serotonin transporter inhibitor (fluoxetine) on evoked concentration and clearance (t_{50}) of octopamine (paired t-test). Values are reported as mean $\pm$ SEM

Drug	Value	Before Drug	After Drug	P value
Nisoxetine, n = 5	t_{50} (s)	1.4 ± 0.1	1.7 ± 0.1	$p = 0.1577$
Fluoxetine, n = 7	t_{50} (s)	1.4 ± 0.1	1.4 ± 0.1	$p = 0.4830$
Nisoxetine, n = 5	Concentration (μ M)	0.16 ± 0.02	0.18 ± 0.01	$p = 0.4840$
Fluoxetine, n = 7	Concentration (μ M)	0.14 ± 0.01	0.20 ± 0.03	$p = 0.0078^{**}$