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Optogenetic Control of Serotonin and Dopamine Release in *Drosophila* Larvae

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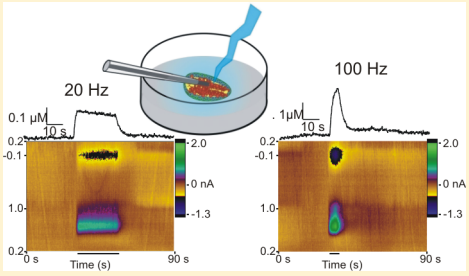
Supporting Information

ABSTRACT: Optogenetic control of neurotransmitter release is an elegant method to investigate neurobiological mechanisms with millisecond precision and cell type-specific resolution. Channelrhodopsin-2 (ChR2) can be expressed in specific neurons, and blue light used to activate those neurons. Previously, in *Drosophila*, neurotransmitter release and uptake have been studied after continuous optical illumination. In this study, we investigated the effects of pulsed optical stimulation trains on serotonin or dopamine release in larval ventral nerve cords. In larvae with ChR2 expressed in serotonergic neurons, low-frequency stimulations produced a distinct, steady-state response while high-frequency patterns were peak shaped. Evoked serotonin release increased with increasing stimulation frequency and then plateaued. The steady-state response and the frequency dependence disappeared after administering the uptake inhibitor fluoxetine, indicating that uptake plays a significant role in regulating the extracellular serotonin concentration. Pulsed stimulations were also used to evoke dopamine release in flies expressing ChR2 in dopaminergic neurons and similar frequency dependence was observed. Release due to pulsed optical stimulations was modeled to determine the uptake kinetics. For serotonin, V_{max} was 0.54 ± 0.07 μM/s and K_m was 0.61 ± 0.04 μM; and for dopamine, V_{max} was 0.12 ± 0.03 μM/s and K_m was 0.45 ± 0.13 μM. The amount of serotonin released per stimulation pulse was 4.4 ± 1.0 nM, and the amount of dopamine was 1.6 ± 0.3 nM. Thus, pulsed optical stimulations can be used to mimic neuronal firing patterns and will allow *Drosophila* to be used as a model system for studying mechanisms underlying neurotransmission.



KEYWORDS: *Drosophila, serotonin, dopamine, optogenetics, FSCV, kinetic modeling*

Optogenetic control of neuronal activity is a new method to selectively activate neurons, with widespread applications in the investigation of brain functions. Channelrhodopsin-2 (ChR2) is a blue-light activated cation channel found in *Chlamydomonas reinhardtii* that can be inserted into specific neurons with genetic manipulations.[1,2] Upon blue light stimulation, ChR2 opens rapidly, and inward flow of cations leads to neuronal excitation.[1,2] In contrast to traditional stimulation methods such as electrical or pharmacological stimulations, optical stimulation of neurons can be controlled with millisecond precision, and this allows targeted activation of a specific type of neuron in one location. In mammals, optical stimulation with ChR2 has been utilized to understand neuronal circuitry that underlies behavior and neurological disorders.[3−6] Optical stimulation is especially useful for small model organisms, such as *Drosophila melanogaster*, the fruit fly, because the bipolar electrical stimulating electrode is large compared to the fly central nervous system (CNS). *Drosophila* are attractive for investigating basic neurobiological mechanisms because of their simple nervous system, evolutionarily conserved neurotransmission pathways, short life cycle, and ease of genetic manipulation.[7] Using cell-specific promoter elements, ChR2 can be inserted into a specific type of neuron in *Drosophila* and those neurons activated by blue light illumination.[8,9]

Structural and functional studies of ChR2 reveal that the channel opens rapidly upon blue light stimulation to generate a large transient photocurrent, and upon continuous illumination the photocurrent decays to a lower steady-state level, that is, it desensitizes.[1,2] When a second pulse is applied after a short dark phase, the transient current component is smaller than the first one.[1] ChR2 reliably drives defined trains of spikes when the frequency of pulsed light is below 40 Hz;[2] however, the correlation between light pulses and cell firing weakens above this frequency.[10−14] Recent studies of ChR2-mediated, optically stimulated dopamine release in vivo reveal U-shaped frequency dependence curves with 40 Hz evoking the maximal signal with 4 ms pulse width, indicating ChR2 kinetics may play a role in stimulated release.[15,16] The pulsed, optically stimulated dopamine release was modeled to determine the kinetics of release and uptake.[15] In contrast to the pulsed optogenetic trains used in mammals, only continuous illumination stimulations have been used to measure evoked neurotransmitter release in *Drosophila*.[8,9,17−19] Long, continuous stimulations do not mimic physiological neuronal firing patterns and the kinetics of release and uptake cannot be directly modeled.

In this study, we characterized the effect of pulsed optical stimulation trains on serotonin and dopamine release in *Drosophila* larval ventral nerve cords. When *Drosophila*

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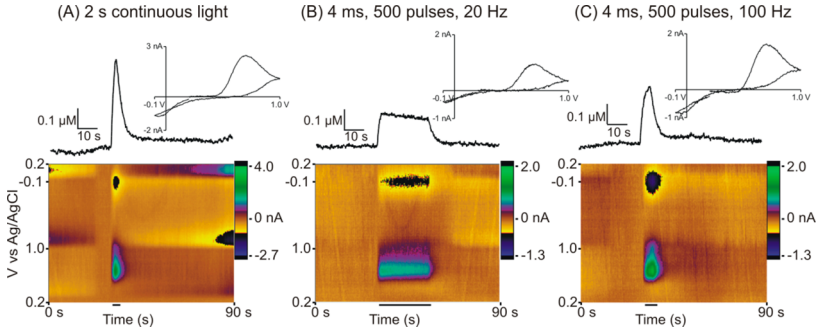

Figure 1. Representative serotonin release evoked by (A) 2 s continuous stimulation, (B) a pulsed stimulation train of 4 ms pulse width, 500 pulses at 20 Hz, and (C) a pulsed stimulation train of 4 ms pulse width, 500 pulses at 100 Hz in the same ventral nerve cord. The bottom panel shows false color plots with time on the *x*-axis, applied voltage on the *y*-axis and background-subtracted faradaic current in pseudocolor. The duration of the stimulation is marked as the black bar below the color plot. The concentration versus time profiles are plotted on top of the color plots by converting the current at the maximal oxidation potential for serotonin to concentration through in vitro calibration. The insets are background-subtracted cyclic voltammograms which confirm that serotonin is detected.

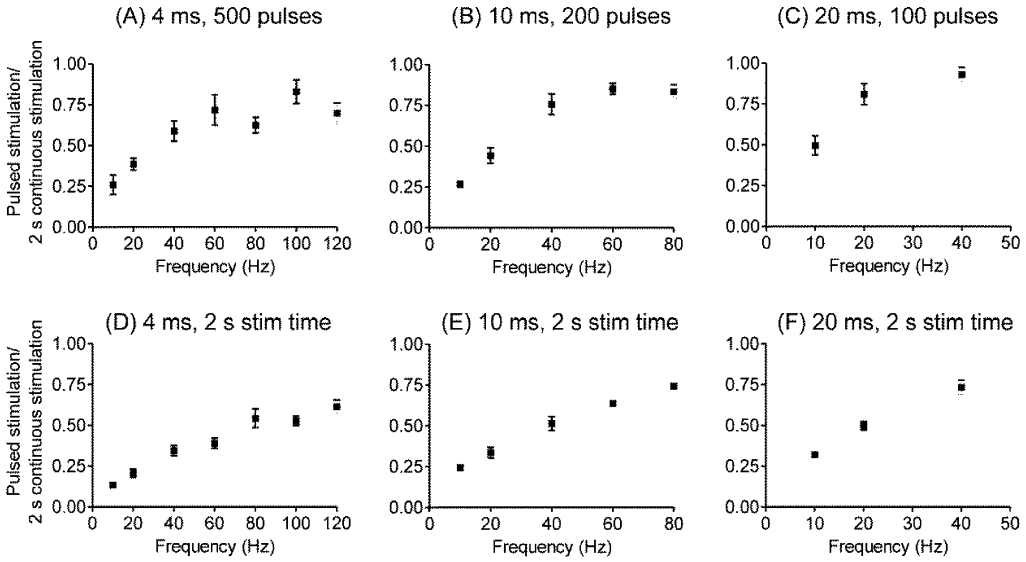

Figure 2. Frequency dependency of stimulated serotonin release for three different pulse widths: 4, 10, and 20 ms. The frequency dependency is tested in two patterns. (A–C) The total amount of light illumination is fixed at 2 s. (D–F) The total simulation duration is fixed at 2 s. It is 20, 40, 80, 120, 160, 200, and 240 pulses for 10, 20, 40, 60, 80, 100, and 120 Hz, respectively. Data are expressed as the ratio of serotonin release by pulsed stimulation to that released by 2 s continuous illumination, which normalizes for different release amounts in different samples. Each panel was evaluated with a one-way ANOVA: (A) $F[6,41] = 11.27$, $p < 0.0001$; (B) $F[4,27] = 35.46$, $p < 0.0001$; (C) $F[2,15] = 16.14$, $p < 0.001$; (D) $F[6,50] = 24.89$, $p < 0.0001$; (E) $F[4,34] = 55.29$, $p < 0.0001$; (F) $F[2,21] = 44.35$, $p < 0.0001$. Data are mean ± SEM, and $n = 5–9$.

serotonergic and dopaminergic neurons are selectively activated in vivo, there are large increases in the amount of firing in the 30–100 Hz range, as well as a smaller increase in the 2–6 Hz range.[20] The pulsed stimulations we tested (10–100 Hz) mimic the faster expected firing rates. We concentrated on serotonin, as serotonin signaling plays a key role in biological processes such as mood and sleep, and the serotonin transporter is a target for many drugs designed to treat psychiatric disorders.[21−23] Release was measured using fast-scan cyclic voltammetry (FSCV) at a carbon-fiber microelectrode implanted in the neuropil of a fly modified using the GAL4/UAS system.[8,9,24] Evoked serotonin release was controlled by different light parameters, including pulse frequency, pulse width, and pulse number. Administration of fluoxetine, a serotonin transporter inhibitor, eliminated the frequency dependence of evoked serotonin. Pulsed stimulations were also conducted in dopaminergic neurons to evaluate the generalizability of our method, and frequency dependent release was observed. The pulsed optical stimulations allowed the release and uptake kinetics to be directly modeled. Our results demonstrate that pulsed optical stimulations combined with FSCV detection can be used to study the effect of different neuronal firing patterns on uptake and release, strengthening the utility of *Drosophila* as a model system for studying mechanisms underlying neurotransmission.

RESULTS AND DISCUSSION

The Shape of Optically Stimulated Serotonin Release is Frequency Dependent. Endogenous serotonin had previously been detected in *Drosophila* larval ventral nerve cords (VNC) using continuous, blue light stimulation. Larvae expressing ChR2 only in serotonergic neurons, which express tryptophan hydroxylase, were used to ensure stimulation specificity.[8,17] With continuous stimulation, the peak concentration increases as a function of stimulation duration until saturation is reached at 10 s.[8] With stimulations longer than 10 s, the peak concentration no

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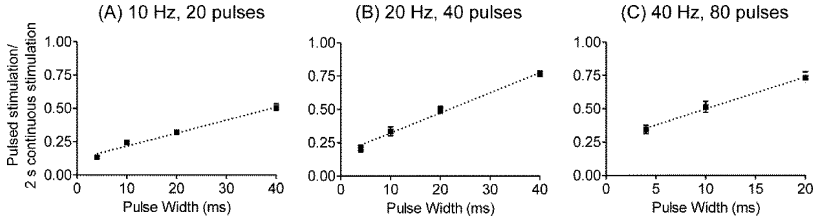

Figure 3. Pulse width dependency of stimulated serotonin release with three different stimulation frequencies: (A) 10 Hz (one-way ANOVA, $F[3,27] = 55.34$, $p < 0.0001$), (B) 20 Hz ($F[3,28] = 77.24$, $p < 0.0001$), and (C) 40 Hz ($F[2,21] = 24.49$, $p < 0.0001$). The total stimulation duration is 2 s. Data are expressed as the ratio of serotonin release by pulsed stimulation to that of the 2 s continuous illumination. Data are mean ± SEM, and $n = 7-8$.

longer increases but plateaus, due to depletion of the releasable serotonin pool and a balance between release and uptake.

To compare pulsed and continuous stimulations, a variety of stimulations were performed with 2 s of light illumination. Figure 1 shows serotonin release in the same larval CNS evoked by 2 s continuous illumination, a 4 ms pulse width stimulation at a low frequency (20 Hz), and a 4 ms pulse width stimulation at a high frequency (100 Hz). Continuous illumination with 2 s of light produces a peak-shaped response (Figure 1A). With the low stimulation frequency, the concentration of serotonin increases rapidly upon stimulation but then reaches a steady-state level (Figure 1B). In contrast, with the high frequency train, the serotonin concentration increases during the entire stimulation to a maximum and then falls to baseline after stimulation stops (Figure 1C), similar to the continuous stimulation (Figure 1A). The maximum concentration evoked by the high frequency train is much higher than by the low frequency, but is less than that evoked by the 2 s continuous illumination.

The Amount of Serotonin Released is Frequency Dependent. The effect of different stimulation train parameters on evoked serotonin release was evaluated. First, the effect of stimulation frequency was tested with three pulse widths: 4, 10, and 20 ms, a range which has been used for study of optical stimulation in rats.[15] This experiment was performed in two patterns, both commonly used in investigating the frequency effect in mammals.[15,25−27] In the first pattern, the total amount of time that the light was illuminated was fixed at 2 s, that is, the product of pulse width and pulse number was kept constant at 2 s (Figure 2A−C). For pulsed stimulations, this means that the total stimulation duration was greater than 2 s. In the second pattern, the total duration of the stimulation was fixed at 2 s; thus, the pulse number was adjusted to keep the product of frequency and pulse number constant at 2 s (Figure 2D−F). Evoked release varies for different samples, so for each fly CNS, serotonin release was first evoked with 2 s continuous stimulation and then the peak concentration for each pulse train was normalized to that of the 2 s continuous stimulation. This ratio is plotted in Figure 2.

The peak concentrations evoked by pulsed stimulation trains were all lower than that evoked by the 2 s continuous illumination (all the ratios in Figure 2 are below 1). While continuous stimulation produced the highest serotonin concentration, the pattern and timing of action potentials are not known. ChR2 can drive irregular spikes under continuous illumination,[1] and the high concentration evoked by the continuous light is likely due to an increased probability of ChR2-driven spikes in a short amount of time, as well as less time allowed for uptake.

For pulsed stimulations, a clear frequency dependence is observed in all the panels of Figure 2 and the general trend is that more serotonin is evoked with higher frequency. There was a significant effect of stimulation frequency on the amount of serotonin released for each graph (one-way ANOVA, all $p < 0.0001$, see figure legend for F values). In the first pattern (Figure 2A−C), the release plateaued at higher frequencies. Bonferroni post-tests showed no significant differences in release for any points over 40 Hz for 4 ms (Figure 2A) and 10 ms (Figure 2B) duration stimulations and over 20 Hz for 20 ms (Figure 2C) stimulations ($p > 0.05$). The peak concentrations were lower in the second pattern compared to the first pattern (Figure 2D−F), due to shorter duration of the applied stimulation train. Bonferroni post-tests in one-way ANOVA analysis showed release plateaued over 80 Hz for 4 ms (Figure 2D) and 60 Hz for 10 ms (Figure 2E), with no plateau observed for 20 ms (Figure 2F). Below the plateau frequency, release increased linearly with stimulation frequency (Figure 2D and E, linear regression, $R^2 > 0.95$).

Next, the effect of pulse width was investigated by using three different frequencies and fixing the total stimulation time at 2 s. All data are normalized to that of 2 s continuous illumination. Evoked serotonin increased as the pulse width increased (Figure 3). A one-way ANOVA revealed a significant main effect of pulse width on the amount of serotonin released at all the selected frequencies ($p < 0.001$). The 40 ms pulse width could not be used with 40 Hz stimulation because the width of the pulse is greater than the period between pulses. At all the selected frequencies, release increased linearly with pulse width (Figure 3, linear regression, $R^2 > 0.98$).

Stimulated serotonin and dopamine release in mammals follows similar patterns to those observed for *Drosophila* larvae. A steady-state response is seen at low frequencies and release is frequency dependent.[28,29] The frequency dependence varies with uptake rate[27] and frequency independent evoked serotonin is found in areas with low uptake rates and in both serotonin transporter knockout and overexpressing mouse brain slices.[25,30] Pulse width dependence has also been reported for electrically stimulated serotonin and dopamine release in anesthetized rats, with greater release observed for wider stimulus pulses.[31]

The frequency dependence of serotonin release might also depend on the ChR2 photocycle kinetics. During blue light stimulation, the photocurrent of ChR2 increases rapidly and transiently and then decays to a lower steady-state level.[1,2] The efficiency of optical stimulation at higher stimulation rates is limited by ChR2 photocycle kinetics,[32−34] but it can also be affected by ChR2 distribution, the tissue properties, and the characteristics of the light pulse.[35] Computations and experiments in the hippocampus have confirmed that higher light irradiance levels, longer light pulses, and increased channel density could evoke action potentials with greater probability.[2,35] In our experiments in *Drosophila* larval VNCs, stimulated serotonin release increased with longer pulse width and with higher light intensity (Figure S1, Supporting Information), consistent with those electrophysiological studies[2,35] as well as

studies of optogenetic control of dopamine release in anesthetized rats and rat brain slices.[15,16]

Electrophysiological studies with ChR2-expressing cells have demonstrated that many cells fail to follow the ChR2-driven spikes above the 40 Hz range in sustained trains[2,10,11] due to slow recovery from inactivation of ChR2[32−34] or host cell-specific properties of potassium and sodium channel activation/inactivation kinetics.[36,37] In anesthetized rats, the optically stimulated dopamine response peaked at 40 Hz with 4 ms pulse width and then decreased, suggesting that ChR2 photocycle kinetics might limit stimulated release.[15] However, in *Drosophila*, with short stimulation trains, the serotonin concentration is linear with frequency up to 80 Hz, indicating that release may not be as dependent on ChR2 kinetics.

The Amount of Serotonin Release is Dependent on the Number of Stimulation Pulses. The effect of pulse number on stimulated serotonin release was tested with a low stimulation frequency (20 Hz, Figure 4A, C) and a high frequency (60 Hz,

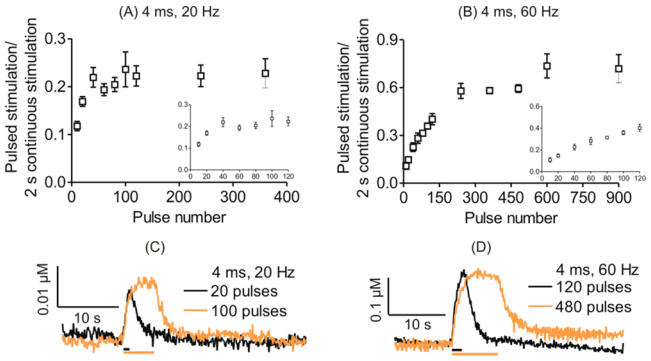

Figure 4. Effect of pulse number on stimulated serotonin release. The effect is tested at two different frequencies (A) 20 Hz and (B) 60 Hz with a 4 ms pulse width. Data are expressed as the ratio between serotonin release by pulsed stimulation and that of the 2 s continuous illumination. Data are mean ± SEM, (A) n = 4−7 and (B) n = 6. Insets are enlarged view of the first 120 pulses. (C) Data from one representative nerve cord at 4 ms, 20 Hz stimulation with two pulse numbers (20 pulses and 100 pulses). A steady-state is achieved with the higher pulse number while not with the lower one. (D) Similarly, data recorded from one nerve cord with 4 ms, 60 Hz stimulation using two pulse numbers (120 pulses and 480 pulses). The duration of the stimulation is marked below the concentration traces (black solid bar for the shorter stimulation and orange solid bar for the longer stimulation). The y scale in (D) is 10 times larger than the y scale in (C).

Figure 4B, D). All stimulations have a 4 ms pulse width and all data are normalized to that of 2 s continuous illumination. Figure 4A and B shows evoked serotonin increases with larger pulse numbers and then plateaus. One way ANOVA analysis showed a significant overall effect of pulse number on the amount of serotonin released for both frequencies ($F[8,45] = 3.01$, $p < 0.01$ for 20 Hz and $F[13,56] = 19.18$, $p < 0.0001$ for 60 Hz). The lower frequency stimulation plateaus at a lower number of pulses than the higher frequency stimulation (40 pulses for 20 Hz versus 240 pulses for 60 Hz). This is illustrated in Figure 4C and D, where the 60 Hz stimulations were peak shaped for 120 pulses but steady-state profile for 480 pulses, while the 20 Hz stimulations had a steady-state profile by 100 pulses. The steady-state serotonin level for 60 Hz stimulations is much higher than that of 20 Hz stimulations (Figure 4C and D). Notably, even for the high stimulation frequency (60 Hz), the plateau concentration was much lower than plateau concentration of long duration continuous stimulations,[8] indicating the plateau concentration at large pulse numbers is a balance between release and uptake, not due to depletion of the releasable serotonin pool.

Serotonin Release and Uptake Kinetics Following Pulsed Optical Stimulation Trains. The most attractive application of pulsed optical stimulations in *Drosophila* is modeling of neurotransmitter release and uptake kinetic parameters directly from individual response curves. The extracellular neurotransmitter concentrations were modeled as a balance between release and uptake. According to the commonly used model of pulsed electrical stimulation in mammals, release is a discontinuous process whereby each stimulus pulse results in a discrete release of neurotransmitter causing an instantaneous increase in extracellular concentration, and uptake is treated as a continuous process that follows Michaelis−Menten kinetics.[28,29,38,39] This model is simplified, particularly because it assumes that the release per pulse is constant. Frequency dependent modulation by autoreceptors might result in different amounts released per pulse.[27,28] The changes in extracellular neurotransmitter levels during and after stimulation were fit using the equation:

$$d[A]/dt = f[A]_p - V_{max}/(K_m[A] + 1)$$

where f is the stimulation frequency (Hz), $[A]_p$ is the concentration of neurotransmitter release per stimulus pulse, V_{max} is the maximal rate of uptake, which is dependent on the number of related transporters, and K_m is the transporter affinity. We used this simplified model to extract basic parameter estimates by fitting the raw data from *Drosophila* larval VNCs.

Figure 5 shows representative serotonin responses evoked by pulsed stimulation trains with a steady state concentration (4 ms,

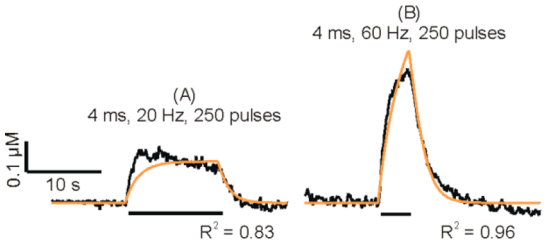

Figure 5. Kinetic modeling of pulsed optically stimulated serotonin release. Data from one representative nerve cord (black lines) with two different stimulation frequencies were fit to a Michaelis−Menten kinetic model to determine the parameters for serotonin release and uptake. Curves with steady-state (A) and non-steady-state (B) were selected for the kinetic modeling. Scale bar is the same for both panels. Simulation lines (orange) were calculated from best-fit parameters ($[serotonin]_p$ = 4.3 nM, V_{max} = 0.48 μM/s and K_m = 0.61 μM). The duration of the stimulation is indicated by the black bar under the curves.

20 Hz) and a nonsteady state concentration (4 ms, 60 Hz) from the same sample, fit with the Michaelis−Menten model. Both curves were fit well with the same set of parameters. The average $[serotonin]_p$ was 4.4 ± 1.0 nM, V_{max} was 0.54 ± 0.07 μM/s, and K_m was 0.61 ± 0.04 μM (35 curves from 28 animals).

The Effects of Uptake Inhibition on Optically Stimulated Serotonin Response. To evaluate the effects of uptake on optically stimulated serotonin response, we blocked serotonin transporter function with 100 μM fluoxetine. Fluoxetine is a selective serotonin reuptake inhibitor in humans. Although it has a lower affinity and selectivity in *Drosophila* compared to mammalian systems,[40,41] fluoxetine has also been used to

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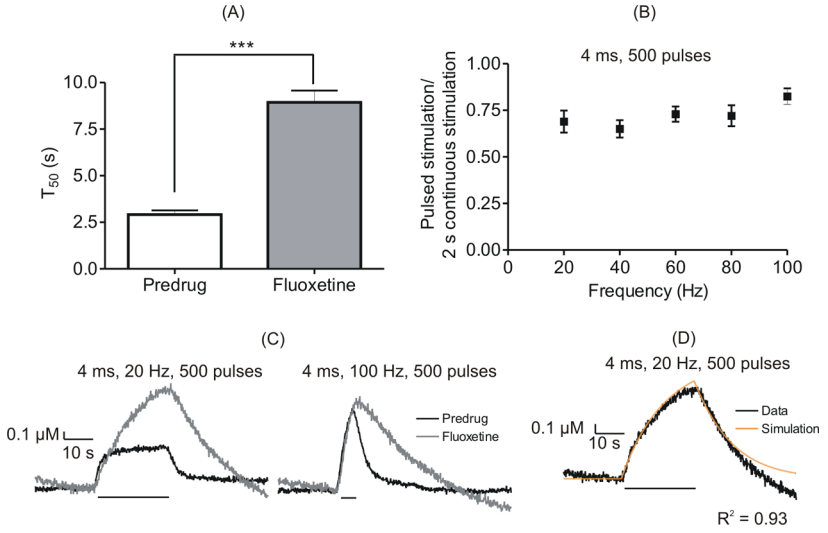

Figure 6. Effects of serotonin uptake inhibitor fluoxetine. (A) With 2 s continuous stimulation, the half decay time (t_{50}) significantly increased 15 min after 100 μM fluoxetine was applied (***$p < 0.001$, paired t test, $n = 15$). (B) Concentrations versus time profiles showing the effect of 100 μM fluoxetine on serotonin release by 4 ms pulse width stimulations at a low (20 Hz) and a high (100 Hz) frequency in the same nerve cord (black bar marks the stimulation duration). (C) Serotonin release is not frequency dependent after 100 μM fluoxetine (data mean ± SEM and $n = 9-11$). Data are expressed as the ratio of serotonin release by pulsed stimulation to that released by 2 s continuous illumination in the presence of fluoxetine. (D) Kinetic modeling of pulsed optically stimulated serotonin release in the presence of 100 μM fluoxetine. Simulation line (orange) to fit the representative data (black) is calculated with the parameters: $[\text{serotonin}]_p$ = 4.4 nM, V_{max} = 0.54 μM/s, and K_m = 6.4 μM, with R^2 = 0.93.

effectively inhibit serotonin reuptake in *Drosophila*.[8,17] The ability of fluoxetine to block uptake in the larval VNC was confirmed by testing 2 s continuous stimulation. An initial 2 s continuous stimulation was performed, then the VNC bathed in 100 μM fluoxetine for 15 min and the same VNC stimulated again. The half decay time (t_{50}) significantly increased after fluoxetine ($n = 15$, paired t test, $p < 0.001$, Figure 6A). Thus, 100 μM fluoxetine effectively blocks serotonin uptake and was used to investigate the effect of uptake inhibition on pulsed stimulations.

Figure 6B shows serotonin release in the same VNC evoked by low frequency (20 Hz) and high frequency (100 Hz) stimulations before and after fluoxetine. After fluoxetine, the time response for both frequencies was slowed and the steady-state response at the low frequency became peak-shaped with a larger peak concentration. The low and high stimulation frequencies produced similar serotonin peak concentrations after fluoxetine; thus uptake regulates concentration and causes the steady-state response for low frequency stimulations. The frequency dependence of evoked serotonin release was investigated in the presence of uptake inhibitor with 4 ms pulses and the total amount of light illumination fixed at 2 s (Figure 6C). There was no significant effect of stimulation frequency on the amount of serotonin released ($F[4,45] = 1.403$, $p = 0.2483$, one-way ANOVA), in contrast to the frequency dependence observed in the low frequency range without an uptake inhibitor (Figure 2A). Our results in *Drosophila* larvae are consistent with results in mouse brain slices where electrically evoked serotonin transients were not frequency dependent in serotonin transporter knockout mice or in wild type mice after uptake inhibition.[30] Furthermore, with the uptake blocker, there was no significant difference on the peak concentration of evoked serotonin release among different stimulation frequencies, indicating ChR2 kinetics was not a limiting factor in our study.

Finally, serotonin release and uptake kinetics were evaluated in the presence of an uptake inhibitor. As a competitive uptake inhibitor, fluoxetine should increase the apparent value of K_m with little effect on release and V_{max}.[28] Thus, we fixed $[\text{serotonin}]_p$ and V_{max} at the predrug values and floated K_m. Figure 6D shows a representative serotonin response in the presence of 100 μM fluoxetine fit with the Michaelis–Menten model. The average of K_m after fluoxetine was 5.8 ± 0.4 μM (20 curves from 10 animals), a 9-fold increase. This increase is of similar magnitude to those observed in the substantia nigra reticulata and the dorsal raphe of rat brain slices after application of fluoxetine.[28]

Dopamine Release and Uptake Kinetics Following Pulsed Optical Stimulation Trains. To evaluate the generality of the parameters for optically induced release, pulsed stimulations were also conducted in *Drosophila* larval VNC expressing ChR2 in neurons containing tyrosine hydroxylase, a dopaminergic synthesis enzyme. Figure 7A shows the frequency dependence of 4 ms pulse width train evoked dopamine release with the total stimulation duration fixed at 2 s. One-way ANOVA analysis showed a significant effect of stimulation frequency ($F[6,21] = 15.21$, $p < 0.0001$). The frequency response of dopamine is similar to that of serotonin (Figure 2D), and the release plateaued after 60 Hz (any two frequencies over 60 Hz are not significantly different, Bonferroni post-tests, $p > 0.05$). Figure 7C shows a representative dopamine response fit well with the kinetic model. The average $[\text{dopamine}]_p$ was 1.6 ± 0.3 nM, V_{max} was 0.12 ± 0.03 μM/s, and K_m was 0.45 ± 0.13 μM (11 curves from 5 animals).

Kinetics Values Compared to Other Studies. The uptake values for *Drosophila* larvae were similar to those previously found in mammals. V_{max} depends on the density of transporter expression, which can vary in different brain regions and in different tissue preparations. The V_{max} reported here for serotonin was similar to that in the substantia nigra reticulata in rat brain slices,[28,39] and the V_{max} for dopamine was similar to that in the nucleus accumbens and prelimbic cortex areas of mice brain slices.[42] The K_m values for both monoamines are of the same order of magnitude as values reported in mammals using electrical stimulations.[15,25,39] Our K_m for serotonin uptake is also

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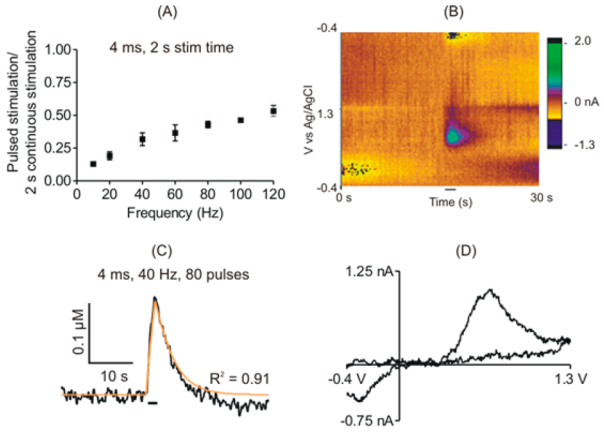

Figure 7. Dopamine release evoked by pulsed optical stimulations. (A) Frequency dependence of stimulated dopamine release with 4 ms pulse width and the total stimulation duration is fixed at 2 s. Data are expressed as the ratio of dopamine release by pulsed stimulation to that of the 2 s continuous illumination. Data are mean ± SEM, n = 4. (B) Representative color plot of dopamine release evoked by a pulsed stimulation train of 4 ms, 40 Hz, 80 pulses. The green and blue areas show the oxidation and reduction peaks of dopamine, respectively. (C) The concentration versus time profile (black) is plotted, and kinetic modeling (orange) was calculated from the parameters: $[\text{dopamine}]_p$ = 2.4 nM, V_{max} = 0.13 μM/s, and K_m = 0.45 μM, with R^2 = 0.91. (D) Background-subtracted cyclic voltammogram confirms that dopamine is detected.

similar to the value of 0.64 ± 0.10 μM obtained from studies expressing *Drosophila* serotonin transporter in transfected cells,[41] but the K_m of *Drosophila* dopamine transporter in transfected cells (4.8 ± 0.4 μM) is an order of magnitude larger than the value we report here.[40] The K_m values may vary with preparations as well, so methods to measure it in intact tissue are valuable. Previous studies of electrically evoked serotonin release in mammals have reported that the magnitude of the $[\text{serotonin}]_p$ was much smaller in vivo compared with values from slice preparations, likely due to tighter control mechanisms in vivo.[28,31,43] The $[\text{serotonin}]_p$ in the fly larval VNC was similar to the in vivo value of $[\text{serotonin}]_p$ in mammals.[43] However, the $[\text{dopamine}]_p$ in flies was much smaller than mammalian $[\text{dopamine}]_p$ values of both electrical stimulated release[29] and optically stimulated release in anesthetized rats with similar stimulation parameters.[15] The expression of ChR2 may vary in *Drosophila* and with the different drivers used to express it, affecting how many action potentials are evoked.

The clearance kinetics of monoamine neurotransmitters in intact *Drosophila* tissue have been estimated previously with electrochemistry. With these methods, the clearance portion of individual curves from stimulated release or exogenously applied monoamine is fit with an exponential decay and the initial rate of clearance used to make a Michaelis–Menten plot.[8,44] The uptake parameters were determined for serotonin after continuous optical stimulations; however, the evoked peak concentrations are not high enough to produce V_{max}. Thus, a full Michaelis–Menten curve is difficult to acquire and the kinetic parameters of clearance might be underestimated.[8] The V_{max} and K_m for serotonin in *Drosophila* larval VNC using this method were 0.17 ± 0.04 μM/s and 0.35 ± 0.08 μM,[8] respectively, both significantly smaller than the values reported here (unpaired t test, $p < 0.0001$ for V_{max} and $p < 0.05$ for K_m). For dopamine, exogenously applied neurotransmitter in *Drosophila* larval VNC was employed to span a wider range of analyte concentrations.[44] A diffusional distortion has been reported with exogenous application, which could lower the apparent rates of uptake and result in a high K_m value,[45] and this was corrected for using *fumin* flies that are dopamine transporter knockouts. The V_{max} and K_m were 0.11 ± 0.02 μM/s and 1.3 ± 0.6 μM, respectively, in that study, which are not significantly different from the dopamine values reported here (unpaired t test, $p > 0.05$). However, this method cannot provide any information about release. Pulsed stimulations are easy to perform, and modeling endogenous release provides an easy way to obtain kinetic values for uptake as well as the amount of neurotransmitter released per stimulation pulse.

CONCLUSIONS

Pulsed optical stimulations can be used in *Drosophila* to evoke neurotransmitter release, and the release is dependent on pulse width, pulse number, and stimulation frequency. With the same number of pulses, low-frequency stimulations have a distinct, steady-state response compared to high frequency patterns, which are peak shaped. Release evoked by pulsed stimulations is lower than that evoked by continuous stimulation because there is more time for uptake between stimuli. The main advantage of using pulsed stimulations is that the release and uptake kinetics of serotonin and dopamine can be estimated without the need to produce large concentrations to reach V_{max}. V_{max} and K_m for serotonin and dopamine in *Drosophila* are similar to their values in mammals. Our results demonstrate that pulsed optical stimulations combined with FSCV detection can be used to measure the effect of firing patterns on release and uptake kinetics, strengthening the utility of *Drosophila* as a model system for studying mechanisms of neurotransmission during behavior and neurological disorders.

METHODS

Chemicals. Chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Solutions were made with Milli-Q water (Millipore, Billerica, MA). Electrode calibrations and *Drosophila* dissections were conducted using a modified Schneider's buffer (15.2 mM $MgSO_4$, 21 mM KCl, 3.3 mM KH_2PO_4, 36 mM NaCl, 5.8 mM NaH_2PO_4, 5.4 mM $CaCl_2$, 11.1 mM glucose, 5.3 mM trehalose, pH = 6.2).

Electrochemical Measurements. Cylindrical carbon-fiber microelectrodes were fabricated in house from T-650 carbon fibers (a gift of Cytec Engineering Materials, West Patterson, NJ) as previously described.[46] Fast-scan cyclic voltammetry data were collected using a ChemClamp 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. Data collection was computer controlled by the TarHeel CV software program (gift of Mark Wightman, University of North Carolina). To detect serotonin, we applied a modified waveform, from 0.2 to 1.0 V, then to −0.1 V and back to 0.2 V versus a Ag/AgCl reference electrode at a scan rate of 1000 V/s with a repetition rate of 10 Hz.[47] To detect dopamine, the electrode was scanned from −0.4 to 1.3 V and back at a scan rate of 400 V/s at 10 Hz. Electrodes were calibrated with 1 μM serotonin or 1 μM dopamine before and after use in situ.

Preparation of Ventral Nerve Cords. Fly stocks were made as described previously.[8,18] Flies containing wild-type channelrhodopsin-2[1,48] with the genotype UAS-ChR2 (a gift from Christian Schroll, Universitat Wurzburg) were crossed to flies expressing Tph-GAL4 (a gift from Jaeson Kim, Korea Advanced Institute of Science and Technology) to generate homozygous lines with a Tph-GAL4; UAS-ChR2 genotype. Flies containing UAS-H134R-ChR2 (Bloomington stock center) were crossed to flies expressing Th-GAL4 (a gift from Jay Hirsh, University of Virginia) to generate homozygous lines with a UAS-H134R-ChR2; Th-GAL4 genotype. The H134R ChR2 single mutant has been shown in previous studies to have increased photocurrent size

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and higher reliability at low-frequency spiking but a drop-off in spike reliability at frequencies above 40 Hz.[12,49] Homozygous, 3-day-old larvae were shielded from light and fed all-trans retinal, mixed with Red Star yeast (Red Star, Milwaukee, WI), for 2−3 days prior to the dissection. The fly dissection and all the measurements were performed at room temperature. The central nervous system of a 5-day-old wandering third instar larva (L3W) was dissected out in modified Schneider's buffer, and the optic lobes were removed by a horizontal cut across the anterior thorax region to yield an isolated ventral nerve cord (VNC).[8] The isolated VNC was adhered neuropil side down onto the bottom of a Petri dish with 3 mL of buffer. The VNC was visualized under a 40× water immersion objective of a microscope (Carl Zeiss Microscopy, LLC), and an electrode was implanted using a micromanipulator into the VNC four to six segments away from the cut edge. The electrode was allowed to equilibrate for at least 5 min before data collection. Thirty seconds of baseline data was collected before each stimulation.

Optical Stimulation. The optical stimulation setup consisted of a 473 nm diode laser (IkeCool Corporation, Los Angeles, CA) with a built-in optical fiber of core diameter 200 μm and 1.5 m in length. The optical fiber was coupled to the fluorescent microscope and the laser beam was focused on the sample via the microscope objective lens. The laser was modulated with the Transistor-Transistor Logic (TTL)-input control port on the laser power supply, which was connected to the breakout box. The TTL input of the laser was driven by electrical pulses controlled by the TarHeel program. The program controlled the frequency, pulse width, and pulse number of the TTL pulses. The Supporting Information contains information on the effect of laser power on stimulation.

Statistics and Data Analysis. Data are presented as mean ± standard error of the mean (SEM) for n number of fly samples, and all error bars are given as SEM. One-way ANOVA with Bonferonni post-tests was used to compare effects among multiple groups. All statistics were performed in GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). Curve fitting for release and uptake kinetics was performed using a nonlinear regression with a simplex minimization algorithm[50,51] and the goodness of fit was described by the square of regression coefficient (R^2). All R^2 values for curve fitting were greater than 0.83. In most mammalian studies, K_m is fixed at an accepted value determined in brain synaptosomes or slices, and the program determines V_{max} and release per pulse.[25,27,28,43] As there is no published K_m value in fly synaptosomes, we chose to float all three parameters. An upper limit of K_m was set as a limitation of the iteration number, which was determined from the highest reported literature values (0.8 μM for *Drosophila* serotonin transporter[8,40,41] and 4.8 μM for *Drosophila* dopamine transporter[40,44]). Thus, the program was stopped either when K_m reached the upper limit or a point where the iteration number kept increasing but the three parameters did not change. The average values of kinetic parameters were obtained from multiple, 2 s stimulation curves with 4 ms pulse width at 4 different frequencies (20, 40, 60, and 80 Hz).

■ ASSOCIATED CONTENT

Supporting Information

Additonal methods and results on the effect of laser power on stimulation. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

N.X. performed the serotonin experiments and E.P. performed the dopamine experiments. N.X., E.P., and B.J.V. conceived of the experiments and wrote the paper.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ChR2, channelrhodopsin-2; FSCV, fast-scan cyclic voltammetry; VNC, ventral nerve cord; TTL, transistor-transistor logic; DAT, dopamine transporter

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