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Quantitative evaluation of serotonin release and clearance in *Drosophila*

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Abstract

Serotonin signaling plays a key role in the regulation of development, mood and behavior. *Drosophila* is well suited for the study of the basic mechanisms of serotonergic signaling, but the small size of its nervous system has previously precluded the direct measurements of neurotransmitters. This study demonstrates the first real-time measurements of changes in extracellular monoamine concentrations in a single larval *Drosophila* ventral nerve cord. Channelrhodopsin2-mediated, neuronal type-specific stimulation is used to elicit endogenous serotonin release, which is detected using fast-scan cyclic voltammetry at an implanted microelectrode. Release is decreased when serotonin synthesis or packaging are pharmacologically inhibited, confirming that the detected substance is serotonin. Similar to tetanus-evoked serotonin release in mammals, evoked serotonin concentrations are 280 – 640 nM in the fly, depending on the stimulation length. Extracellular serotonin signaling is prolonged after administering cocaine or fluoxetine, showing that transport regulates the clearance of serotonin from the extracellular space. When ChR2 is targeted to dopaminergic neurons, dopamine release is measured demonstrating that this method is broadly applicable to other neurotransmitter systems. This study shows that the dynamics of serotonin release and reuptake in *Drosophila* are analogous to those in mammals, making this simple organism more useful for the study of the basic physiological mechanisms of serotonergic signaling.

Keywords

FSCV; *Drosophila*; channelrhodopsin; SERT; cocaine; reserpine; PCPA

Introduction

Serotonin and the serotonin transporter (SERT) are of interest due to their clinical relevance (Murphy et al., 2004). Drugs targeting SERT are used in the treatment of depression and other

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psychiatric illnesses. Polymorphisms affecting serotonin degradation and SERT expression have been associated with depression and anxiety (Lasky-Su et al., 2005; Serretti et al., 2006). The basic mechanisms of serotonergic signaling are highly conserved from mammals down to *C. Elegans* (Nichols, 2007). *Drosophila melanogaster*, because of its simple nervous system, short life cycle, and ease of molecular and genetic manipulation is an attractive alternative to research on mammals (Bier, 2005). While progress has been made in understanding the effects of serotonin on *Drosophila* behavior (Yuan et al., 2005) and morphology (Sykes and Condrón, 2005), measurements of real-time serotonin release and reuptake have been hampered by a lack of analytical tools.

Quantitative evaluation of serotonin levels in the fly larvae has heretofore relied on whole-brain homogenization followed by analysis with high performance liquid chromatography (Park et al., 2006) or capillary electrophoresis (Paxon et al., 2005). Fast scan cyclic voltammetry (FSCV) at carbon-fiber microelectrodes has been well characterized for use in mammals (Phillips et al., 2003) and provides numerous advantages. The diameter of the electrodes (7 μM) makes them amenable to implantation in the small larval fly nervous system. Implanted electrodes sample from the extracellular fluid, thus allowing direct measurement of the functional serotonin pool. Measurements are collected every 100 ms, allowing rapid detection of release and clearance (Bunin and Wightman, 1998). FSCV provides a cyclic voltammogram (CV), characteristic of the analyte detected, that aids in analyte identification (Garris et al., 2003).

Here we utilize a novel application of FSCV to quantify changes in extracellular monoamines in the isolated fly larval ventral nerve cord (VNC). Channelrhodopsin-2 (ChR2)-mediated stimulation produces neuron-specific induction and physiologically-relevant release. Pharmacological agents that inhibit serotonin synthesis and packaging confirm the measured compound is serotonin and that release is vesicular. We show that the dynamics of serotonin release and reuptake in *Drosophila* are analogous to those in mammals and that transport is involved in the clearance of serotonin from the extracellular space. The ability to monitor release and transporter function in real-time significantly strengthens the utility of *Drosophila* as a model system for studying the basic mechanisms underlying neurotransmission.

Materials and Methods

Instrumentation and electrochemistry

Carbon-fiber microelectrodes were manufactured from single T-650 carbon fibers, 7 μm in diameter (Cytec Engineering Materials, West Patterson, NJ), as previously described (Venton et al., 2006). A Dagan ChemClamp potentiostat was used to collect electrochemistry data (Dagan, Minneapolis, MN; custom-modified). Data acquisition software and hardware were the same as described by Heien et al (Heien et al., 2003). For dopamine detection, the electrode potential was continuously scanned from -0.4 to 1.3 V and back at 600 V/s every 100 ms, even when data was not being collected. For serotonin detection, we applied a modified waveform, from 0.2 V to 1.0 V, then to -0.1 V then back to 0.2 V at a slew rate of $1,000$ V/s every 100 ms (Jackson et al., 1995). A silver-silver chloride wire was used as a reference electrode.

Electrodes were calibrated with 1 μM serotonin or 1 μM dopamine prior to and after use *in situ*. A small percentage of electrodes were discarded because they exhibited a signal to noise ratio of less than 100 or were insufficiently sensitive, with less than 6 nA of oxidative current for 1 μM serotonin or dopamine.

Fly stocks

Flies containing Channelrhodopsin-2 (a gift from Christian Schroll, Universitat Wurzburg) were crossed to flies expressing TH-GAL4 or Tph-GAL4 (a gift from Jaeson Kim, Korea Advanced Institute of Science and Technology) to generate homozygous lines with the following genotypes: Tph-GAL4; UAS-ChR2 and UAS-ChR2; TH-GAL4. Canton S (CS) flies, used as a control, were obtained from Bloomington stock center (<http://flystocks.bio.indiana.edu/>). Larvae were allowed to feed on yeast supplemented with 10 mM all-trans retinal (Sigma-Aldrich, St. Louis) for 2–3 days prior to the initiation of experiments while protected from light.

Preparation of ventral nerve cords (VNCs)

5 day old larval brains were dissected in modified Schneider's insect media (15.2 mM MgSO_4 , 21 mM KCl, 3.3 mM KH_2PO_4 , 53 mM NaCl, 5.8 mM NaH_2PO_4 , 5.4 mM CaCl_2 , 11.1 mM Glucose, 5.3 mM Trehalose, pH = 6.2). The composition of this media allows the long-term culture of isolated VNCs and the electrochemical detection of serotonin. The optic lobes were removed by means of a horizontal cut in the anterior-most portion of the VNC, which was then placed, neuropil side down, onto the bottom of a petri plate in 3ml fresh buffer. The VNC was visualized under a 40X water immersion objective and an electrode was inserted using a micromanipulator to a distance of 4–6 segments away from the cut edge. The manipulator was angled so as to place the electrode within the middle portion of the neuropil. Experiments were not initiated until at least 5 min after electrode implantation to allow the electrode background current to stabilize.

Data collection

Data collection was initiated no later than 50 minutes following VNC isolation. No significant changes in signal peak height or time to half maximal signal decay resulted from changes in wait time within this window (see supplemental figure 1). After the collection of at least 30 seconds of baseline data, VNCs were exposed to 10 seconds of intense blue light to stimulate release. The light source was a 10W halogen microscope bulb with a standard fluorescein emission filter (450 – 490 nm) that was manually-switched. VNCs were subsequently allowed to rest in the dark for 5–10 minutes before the next stimulation. Peak height remained stable when 10s long stimuli were performed 5 min apart (data not shown) and this inter-stimulation time was used for variable length stimulation experiments. The following drugs were purchased from Sigma-Aldrich: Reserpine, Fluoxetine hydrochloride, 4-Chloro-DL-phenylalanine (PCPA), and cocaine hydrochloride. In experiments involving pharmacological manipulations, VNCs were dissected and incubated in the presence of drug for 20–30 minutes before stimulation to allow time for drug diffusion.

Data analysis

Electrochemical data was analyzed using Tar Heel CV software (Heien et al., 2004). Data were visualized as a background-subtracted cyclic voltammogram (CV), a concentration vs time trace, or a 3D color plot showing all the data. For a detailed description of monoamine detection by FSCV, see supplemental Fig. 2. All CVs and color plots shown in this paper have been background subtracted by averaging 10 scans collected 1 second before blue light exposure. Signal CVs were collected approximately 1 second after the cessation of blue light exposure. CVs were used to identify the voltage corresponding to the maximum serotonin or dopamine oxidation peaks. The current at this voltage was converted to serotonin concentration using post calibration data for that electrode, and these changes in concentration over time are plotted as the signal trace.

To aid in electrochemical identification, the CV obtained during electrode calibration was compared to the CV from the VNC. The mean R^2 for a linear regression fit between calibration and signal CVs for 10 electrodes was 0.82 ± 0.02 . The reasons for differences between calibration and VNC data include different ionic concentrations inside the tissue and a calibration method that results in small changes in the background capacitance due to changing fluid levels. Additionally, minor shifting apart of the oxidation and reduction peaks occurs as a result of slightly slower kinetics *in situ*. Less than 5 % of samples were excluded because of poor electrode placement or electrode drift. VNCs were excluded if the shape of the background current in the VNC did not match that of the background current in the buffer. This happened when the electrode was not implanted in the neuropil or remained attached to the glial outer layer and resulted in a background that was more triangular shaped than normal. VNCs were also excluded if the electrode had severe drift because drift can cause errors in measuring peak duration. Severe background drift was defined as a change in baseline current of more than 1.5 nA in 100s. Greater amounts of drift and larger peak shifts were observed when using the dopamine waveform. Statistical analysis of pooled data including two-tailed student t tests was conducted using Excel and InStat software. Curve fitting was done in GraphPad Prism.

Results

Characterization of ChR2-mediated serotonin and dopamine release in the fly

To provide neuron-specific stimulation, we expressed ChR2, a blue light-activated, cation-selective ion channel from the green algae *Chlamydomonas reinhardtii* (Schroll et al., 2006). ChR2 allows neural excitation on a millisecond timescale and provides single action potential control of neuronal signaling. Flies containing ChR2 under the control of a GAL4 binding upstream activator sequence (UAS) were crossed to Tph-GAL4 or TH-GAL4 “driver” lines to provide serotonergic or dopaminergic-specific expression, respectively. Tryptophan hydroxylase (Tph) and Tyrosine hydroxylase (TH) are the rate-limiting enzymes in the biosynthesis of serotonin and dopamine (Zhang et al., 2004). Electrodes were implanted into the neuropil of isolated, intact *Drosophila* VNCs from wandering third instar larvae, which exhibit a mature, fully-developed serotonergic system (Sykes and Condron, 2005).

To detect serotonin, we applied a modified waveform, from a holding potential of 0.2 V to 1.0 V, then to -0.1 V and back to 0.2 V at 1000 V/s every 100 ms. This waveform was previously optimized for the sensitive detection of serotonin, provides a 10-fold greater electrode sensitivity for serotonin over dopamine, and alleviates electrode fouling by oxidized serotonin (Jackson et al., 1995). Untreated electrodes were used instead of Nafion coated electrodes to facilitate fast electrode response times. Figure 1a–c shows that a ten second duration blue-light stimulation of a Tph-GAL4; UAS-ChR2 VNC elicits serotonin release. The green and blue areas on the color plot correspond to the oxidation and reduction of serotonin (Fig. 1a). The background-subtracted CV (Fig. 1b) confirms that serotonin was detected because the peak locations for the larval CV (black line) are similar to the 1 μ M serotonin calibration CV (blue line). The concentration vs time trace shows that changes in serotonin are time-locked to the stimulus (Fig. 1c).

Serotonin release was not detectable in any of the control VNCs including those from the parental strains UAS-ChR2 and Tph-GAL4, as well as Canton S (CS). For example, in Fig. 1d, the color plot from a CS VNC shows minor fluctuations across voltages during the stimulation, with the largest change occurring at the switching potential (1.0 V). No characteristic oxidation or reduction peaks for serotonin are apparent in the CV (Fig. 1e). The concentration vs. time trace, at the potential for serotonin oxidation in Fig. 1f shows that the minor noise fluctuation corresponds to about 70 nM serotonin. Pooled data ($n = 4$) shows that on average, this error is about 11 % of the normal peak serotonin detected.

To detect dopamine, we applied a standard, triangle waveform from -0.4 to 1.3 V and back at 600 V/s every 100 ms. This waveform exhibits enhanced electrode sensitivity to dopamine (Heien et al., 2003). Stimulation of a TH-GAL4; UAS-ChR2 VNC evoked dopamine release, as evident in the color plot (Fig. 1g) and signal CV (Fig. 1h). Dopamine release was also time-locked to the stimulation (Fig. 1i). No dopamine was observed in a control VNC from a larva expressing only TH-gal4 but no UAS-ChR2 (Fig. 1j). As seen with the serotonin control experiments, minor changes are observed across voltages but no peaks corresponding to the oxidation or reduction peaks of dopamine are visible (Fig. 1k). This results in an error of about 60 nM (Fig. 1l).

The GAL4-UAS system allows specific stimulation of one type of neuron, so release should be predominantly either dopamine or serotonin. While distinguishing serotonin from dopamine solely based on electrochemistry is difficult, previous studies have used cyclic voltammetry for codetection of serotonin and dopamine (Zhou et al., 2005). The dopamine CV has a greater separation between oxidation and reduction peaks and the positions of the dopamine and serotonin reduction peaks are far enough apart to allow them to be separated. Consistent with this, no serotonin-specific reduction peaks were observed after stimulation of VNCs expressing ChR2 in dopaminergic neurons. Because the electrode is more sensitive to serotonin, the reduction peak would be expected to be detected if serotonin had been present.

The peak serotonin concentration detected varies with the duration of blue light exposure (Fig. 2). Traces from a representative VNC show the relative size of the peaks elicited by 2, 5, 10, or 30 s stimulation (Fig. 2a). These stimulations elicited $280 - 640$ nM serotonin. The two second-long stimulation elicits roughly half the peak serotonin concentration as seen with 10 s. The peak concentration for a 10 s stimulation is about equal to that using 30 s, although the duration of the signaling is longer with the longer stimulation. Therefore, uptake probably acts to clear some of the serotonin during the stimulation, leading to a steady-state concentration. Because the light source was manually operated, stimulation lengths less than 2s were difficult to achieve. Pooled data shows that maximal serotonin concentration appears to reach a plateau at 10 s (Fig. 2b) and the peak height for 30 s stimulations was not significantly different than 10 s stimulations. We chose to use 10 s long stimulations for the rest of the experiments that characterize serotonin release and clearance.

Release is due to vesicular serotonin

To quantify evoked signals, peak height (maximal concentration) and the time for half maximal signal decay (t_{50}) were calculated. A diagram of the measured parameters is shown in Fig. 3a. When VNCs are dissected in buffer, signals remain relatively stable for 90 min when 10s long stimulations are performed 10 min apart (Fig. 3b). Pooled data shows the relative stability of both the peak height and t_{50} over the course of 8 stimulations (Fig. 3c,d).

Although electrochemical evidence strongly suggests that the observed signal is serotonin, we chose not to rely on electrochemical identification as the sole evidence of serotonin detection. We therefore conducted experiments to verify the nature of the analyte by pharmacologically disrupting the synthesis or packaging of serotonin into vesicles. When VNCs are incubated for 30 min in the serotonin synthesis inhibitor PCPA, the peak height decreases for later stimulations. Representative traces from a VNC dissected in $100 \mu\text{M}$ PCPA are superimposed in Fig. 4a to highlight the decreasing peak height. Pooled data shows that while the initial stimulation is similar to controls, the peak height with PCPA decreases to $50 \pm 3\%$ of the initial peak height by the fourth stimulation (Fig. 4b). The decrease in signal with synthesis inhibition confirms the identity of the electroactive species as serotonin.

To test if release was from vesicular serotonin, the packaging of serotonin into vesicles by the vesicular monoamine transporter (VMAT) was inhibited with reserpine. Blue light stimulation

of VNCs incubated in 100 μ M reserpine for 30 minutes did not elicit detectable serotonin release. Color plots and CVs from reserpine-incubated VNCs did not show any peaks consistent with serotonin detection (supplemental Fig. 3). A representative trace from a VNC incubated in reserpine is superimposed on one from a control VNC lacking ChR2 expression to highlight that the small change is due to noise seen in all VNCs and not serotonin (Fig. 5a). Pooled data (Fig. 5c) show that peak height with reserpine is not significantly different from control VNCs lacking ChR2 expression. These data show that serotonin release is vesicular.

Characterization of serotonin clearance in the fly

We expect that released serotonin is eliminated primarily through reuptake by SERT, since *Drosophila* do not contain significant levels of monoamine oxidase, the enzyme responsible for breakdown in higher animals (Paxon et al., 2005). To test this hypothesis, we blocked transporter function with 10 μ M cocaine or 10 μ M fluoxetine. After reuptake inhibition, serotonin clearance is slower and signal takes longer to return to baseline (Fig. 5b). No additional peaks are detected after either cocaine or fluoxetine (supplemental figure 3). Pooled data show that transporter inhibition does not affect the peak evoked serotonin concentration (Fig. 5c) but does significantly increase t_{50} by about 3 times (Fig. 5d). This parameter therefore correlates to the rate of serotonin reuptake.

Kinetic analysis of serotonin uptake

To characterize serotonin clearance in *Drosophila*, the kinetic constants K_T and V_{max} were estimated. These parameters are measures of transporter affinity and number of uptake sites, respectively. Constants were estimated using previous methods that assume all clearance is due to uptake (Sabeti et al., 2002; Daws et al., 2005; John and Jones, 2007). Briefly, the decay portion of each peak was fit with a single exponential decay function using nonlinear regression analysis:

$$A(t) = A_{max} * e^{-k * t}$$

where A is the serotonin concentration at a given time, A_{max} is peak height, and k is the first-order rate constant. A representative trace superimposed with the exponential fit (yellow line on Fig. 6a) shows that the exponential decay function closely follows the actual signal. Optimal fit (maximized R^2 value) was achieved when data was fit from the time stimulation ended until $t=22-30$ s later depending on stimulation length. This time corresponded to a signal decay of about 80%, the same cutoff used in previously published work (Sabeti et al., 2002).

Initial velocity of serotonin clearance (V) was calculated from the equation $V = k * A_{max}$. A plot of V versus peak concentration (Fig. 6b) is obtained by pooling data from multiple peaks obtained during variable length stimulations (up to 10 s). Data was arranged by signals of increasing peak height and then placed into bins according to a pseudo-geometric progression. Each point therefore has error bars in the x and y directions ($n = 3-12$ peaks per point). A four-parameter logistic equation was fit to this plot, consisting of a sigmoid function with a variable slope and the baseline constrained to zero. The equation was $V = V_{max} * A_{max}^H / (K_T^H + A_{max}^H)$, where K_T is the serotonin concentration corresponding to half of V_{max} . From this equation, V_{max} was estimated as 170 ± 40 nM/s and K_T as 350 ± 80 nM for our data. The R for this fit was 0.86. This model predicts that a maximal rate of uptake would be achieved at concentrations greater than 4 μ M. A plot of k , the exponential constant, versus evoked concentration (Fig. 6c) is obtained from the same data set. k is proportional to V_{max}/K_m and should be constant until near V_{max} , when it should decrease. At the smallest concentration we observe large errors and k appears high. At higher concentrations k is relatively stable but exhibits a slight downward trend.

When VNCs were incubated in cocaine, k is significantly decreased 4.8 fold to 0.055 ± 0.004 ($p < 0.0001$, student t-test, 2 tailed, $n = 11-19$). In fluoxetine, k is significantly decreased 4.4 fold to 0.06 ± 0.004 ($p < 0.0001$, student t-test, 2 tailed, $n = 11-15$). The k values derived from peaks in fluoxetine-incubated VNCs were not significantly different from the k values for cocaine-incubated VNCs (student t-test, 2 tailed, $p = 0.4$).

Discussion

Knowledge of extracellular serotonin signaling in the fly has heretofore been limited by the lack of rapid detection techniques. We have optimized a novel combination of methods to induce and measure rapid changes in serotonin levels in the isolated fly VNC. These are the first real-time measurements of endogenous monoamine changes in *Drosophila* larvae. Fast-scan cyclic voltammetry at implanted carbon-fiber microelectrodes provides subsecond temporal resolution for detection of serotonin changes (Jackson et al., 1995). Expression of ChR2 allows neuronal type-specific depolarization as opposed to global stimulation typical of electrical or ionic stimulation paradigms (Parrish et al., 2006; Schroll et al., 2006; Zhang et al., 2007). When larval VNCs expressing ChR2 under the control of TH-gal4 or Tph-gal4 are exposed to blue light, release of endogenous dopamine or serotonin is detected, respectively. The observed concentration changes are coincident with the stimulation, therefore the ChR2-mediated depolarization is fast and neurotransmitter is released primarily during the stimulation. The CV and pharmacological agents that affect release allow us to confirm serotonin is being detected. Michaelis-Menten uptake parameters for SERT were estimated in intact *Drosophila* tissue for the first time.

Serotonin release is vesicular and dependent on serotonin synthesis

To confirm pharmacologically that the observed signal results from serotonin and not the release of another electroactive substance, VNCs were incubated with the serotonin synthesis inhibitor PCPA (Dasari et al., 2007). While VNCs dissected in buffer show relatively stable signals across repeated stimulations performed 10 minutes apart, PCPA-incubated VNCs show a steady decrease in amplitude, resulting in a decrease to 47% of the initial value after 4 stimulations. The decrease in release by PCPA, in addition to the cyclic voltammogram shape, confirms that serotonin release is being measured.

Serotonin efflux predominantly occurs, in mammals and leech, through vesicle exocytosis and not through reverse transport by SERT (Fon et al., 1997; Wang et al., 1997; Bruns et al., 2000). Our work shows that release in the fly is also vesicular because it is dependent on the packaging of serotonin into secretory vesicles by VMAT. When VMAT is inhibited by reserpine, serotonin release is not observed. The rate at which serotonin release capacity decays in the presence of reserpine is rapid, with complete elimination of the serotonin signal observed within 30 minutes of drug exposure. This is different from the pattern of serotonin release in the presence of PCPA, where the initial peak was not significantly different from that in buffer. When only synthesis is inhibited, recycling of serotonin by the transporter may be able to maintain a vesicular population. The severe serotonin depletion observed after reserpine incubation may also be due to non-specific release of vesicular serotonin. However, this proposed mechanism of reserpine action is not well characterized and it is unclear if it occurs in the fly (Pletscher et al., 1955; Reimann and Schneider, 1998).

Characterization of serotonin release in the fly

Channelrhodopsin2-mediated stimulation elicits release of endogenous serotonin in physiologically relevant concentrations. Because serotonin release has not been previously characterized in the fly, we performed calculations to estimate the number of vesicles needed to produce extracellular serotonin concentrations similar to those measured. We assumed that

each release site would synchronously release one vesicle into the extracellular space and that reuptake would not decrease the size of the resulting peak. The wandering third instar *Drosophila* VNC neuropil contains an average of 12 varicosities per $1,000 \mu\text{m}^3$, which are putative serotonin release sites (Chen and Condron, 2008). If every varicosity released one vesicle containing 4,700 molecules of serotonin (Bruns and Jahn, 1995), the resulting concentration in the sampling volume would equal 450 nM. We detected serotonin concentrations ranging from 280 to 640 nM following 2s to 30s of blue light exposure. These values are consistent with those obtained by electrical stimulation in mammalian substantia nigra reticulata (SNr) slices, where values ranging from 54 to 640 nM were detected for 1 to 20 electrical pulses (Bunin and Wightman, 1998). The detected concentrations range from 64% to 142% of the concentration expected from synchronous single quantum release in the fly. Therefore, on average, during maximal stimulation each varicosity releases 1.5 vesicles.

While the above calculation shows that the values obtained after blue light stimulation are within the physiologically-relevant range, it does not allow us to make any inferences about the release at specific varicosities. The electrode, because of its size, samples from many varicosities (Wightman, 2006). Some varicosities may not be stimulated, so the actual amount released per activated varicosity may be greater. Additionally, reuptake acts to decrease the observed peak concentration, likely causing an underestimation of actual release (Bunin and Wightman, 1998). This error would be most severe for 30s long stimulations and this balance of release and reuptake would explain why the peak shape plateaus during the stimulation.

Effect of transporter inhibition on serotonin clearance

We expect that released serotonin is cleared primarily through reuptake. To test this hypothesis, we blocked transporter function with cocaine or fluoxetine. Both drugs lengthened the time for serotonin to decay back to baseline, an indication that uptake is involved in serotonin clearance. In humans, cocaine shows nearly equal affinity for SERT and DAT, while in the fly cocaine has 6x greater affinity for SERT (dSERT $K_i = 0.47 \mu\text{M}$, versus dDAT $K_i = 2.7 \mu\text{M}$ (Porzgen et al., 2001)) (Corey et al., 1994). Fluoxetine is a typical selective serotonin reuptake inhibitor in humans, but it has a more limited selectivity in *Drosophila* (dSERT $K_i = 73 \text{ nM}$ vs dDAT $K_i = 240 \text{ nM}$ (Porzgen et al., 2001)). Although serotonin can be taken up by the dopamine transporter, the published K_m of dDAT for serotonin ($43 \mu\text{M}$) is 86 times larger than that of dSERT ($490 - 637 \text{ nM}$) (Corey et al., 1994; Porzgen et al., 2001). The peak concentrations observed in these experiments were 280–640 nM. This is very close to the K_m for SERT but over 67x less than that for DAT. Assuming that SERT and DAT transport serotonin at similar rates, at the observed concentrations, DAT would transport an order of magnitude less serotonin than SERT. It is therefore unlikely that DAT-mediated reuptake makes a sizeable contribution to the clearance of serotonin.

Kinetic analysis of serotonin uptake in the fly

Michaelis-Menten modeling can be used to describe parameters for uptake. V_{max} is the maximal rate of uptake, which is dependent on the number of transporters. K_T is the serotonin concentration at which the clearance rate is half maximal. These are the first estimates of uptake parameters in intact tissue in *Drosophila*. Our estimated K_T for serotonin clearance in the fly is $350 \pm 80 \text{ nM}$. K_T should be of the same order of magnitude as K_m and this is close to previously published K_m values of 490 and 637 nM for dSERT obtained from studies using transfected cells (Corey et al., 1994; Porzgen et al., 2001). K_m measurements in mammals have ranged from 170 nM (Bunin and Wightman, 1998) to $5.4 \mu\text{M}$ (Daws et al., 2005). The estimated V_{max} is $170 \pm 40 \text{ nM/s}$, similar to the 198 nM/s observed for mammalian SERT in the hippocampus (Daws et al., 2005). While our kinetic parameters are just estimates, as the elicited peak heights are smaller than the concentration necessary to produce V_{max} , they show that the parameters in the fly are of similar to those in mammals. Future studies could use the application

of exogenous serotonin to produce larger serotonin concentrations and provide a better estimate of $V_{\max}$. A caveat to these calculations, including the previously published values, is that they do not take diffusion into account. While the values derived here can be compared to similarly-derived values, they may overestimate the actual $V_{\max}$ for dSERT because all clearance is assumed to be due to uptake. The role of diffusion in the clearance of serotonin could be investigated by monitoring the clearance of a similarly-sized but non-transported molecule and represents a key area for future research.

Acute transporter inhibition results in a significant increase in t_{50} with no significant effect on serotonin release. This finding is consistent with a key role of reuptake in the clearance of serotonin and with results from electrical stimulation in brain slices (Bunin and Wightman, 1998). When transporter activity is inhibited with cocaine or fluoxetine, k is decreased 4.8 fold or 4.4 fold respectively. The k values for fluoxetine were not significantly different from cocaine indicating that the drugs had a similar effect on serotonin clearance. Assuming these are competitive uptake inhibitors and that $V_{\max}$ remains the same, a change in k would be inversely proportional to a change in K_m . Therefore, both drugs increase apparent K_m over 4-fold. These changes are of similar magnitude to the 5–6 fold changes in K_m that have been observed after cocaine or fluoxetine incubation in mammalian SNr slices (John and Jones, 2007).

Conclusions

Monoamine changes have been routinely measured in mammals (Phillips et al., 2003). However, powerful genetic tools are available for *Drosophila* which, combined with our technique, would allow large scale genetic analysis of neurotransmitter dynamics. For example, human polymorphisms affecting SERT expression are associated with depression, anxiety, and efficacy of antidepressants (Murphy et al., 2004). Genetic screens in the fly could help identify genetic elements critical for SERT regulation of serotonin. While this study focused on characterizing serotonin concentrations, we also demonstrated the use of our technique to monitor dopamine. Extension of this technique to encompass other neuronal types is possible because FSCV can detect other electroactive neurotransmitters and enzyme-based biosensors could be used for other neurotransmitters. This study shows that the dynamics of serotonin release and reuptake in *Drosophila* are analogous to those in mammals, making this simple organism more useful for studying the basic physiological mechanisms of neurotransmission.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1.

Characterization of evoked serotonin and dopamine signals. Blue light stimulation of isolated larval *Drosophila* VNCs elicits depolarization in neurons expressing ChR2. Left column: Color plots with dashed white line denoting oxidation potential utilized for signal trace. Duration of blue light exposure (10 s) indicated by blue bars. Color plots are scaled so the maximum oxidative current corresponds to 750 nM monoamine. Middle: The CVs verify the compound being detected. The CV from the fly (black line) is compared to that obtained during electrode calibration with 1 μ M monoamine (blue line). The asterisks identify the oxidation and reduction peaks, which correspond to the green and blue areas in the color plots, respectively. Right: Signal traces show neurotransmitter concentration changes over time. Currents were converted

to concentration based on electrode calibration values. (a–c) Serotonin release from a VNC expressing ChR2 under the control of Tph-GAL4. The color plot (a) and CV (b) exhibit serotonin-specific peaks as evident by the signal CVs match to the calibration CV. The signal trace (c) shows the serotonin peak is time-locked to the stimulus. Data from a control VNC lacking ChR2 expression shows minor fluctuations throughout the color plot (d) upon blue light exposure but the CV (e, black line) does not show any neurotransmitter specific peaks. (f) Trace shows a small error fluctuation in current. (g–i) Dopamine release from a VNC expressing ChR2 under the control of TH-GAL4. Note a different voltage waveform was used than for serotonin. The color plot (g) and CV (h) verify that dopamine was detected. Dopamine release is also time-locked to the stimulation (i). Data from a control VNC lacking ChR2 expression shows changes similar to those observed with the other waveform. Minor fluctuations are present at all voltages during the stimulation (j) but the CV (k) does not show any neurotransmitter specific peaks. (l) Trace shows a small fluctuation in current.

Fig. 2.

Evoked peak serotonin concentration varies with blue light stimulus duration. (a) Representative traces showing the effect of different stimulation lengths (2, 5, 10, and 30s) on peak height in the same sample. (b) Pooled data (mean $\pm$ SEM, $n = 6$ samples) shows an increase in peak height with increasing duration of blue light exposure. Peak height appears to reach a plateau after 10s; peak height at 30s is not significantly different from that at 10s ($p = 0.78$, student t-test, 2 tailed).

Fig. 3. Stability of serotonin signal. (a) Diagram of measured parameters. Peak height, which is the maximal concentration change from baseline, and time to half maximal signal decay (t_{50}), which is the time from the end of the blue light stimulation until the signal decays to half its maximal value, were calculated. (b) Representative traces from same VNC showing repeated stimulations. All data are 10s long stimulations performed 10 min apart. (c,d) Pooled data (mean $\pm$ SEM, $n = 4$) from VNCs dissected in buffer showing that t_{50} (c) and peak height (d) remain relatively stable over 8 stimulations repeated 10 min apart.

Fig. 4.

Effect of PCPA on serotonin release. When VNCs are dissected in the serotonin synthesis inhibitor PCPA, serotonin release decreases with repeated stimulation. All data is from 10 s long stimulations performed 10 min apart. (a) Representative traces from a VNC that has been incubated in PCPA, superimposed to show progressive decrease in peak height. (b) Pooled data (mean $\pm$ SEM, $n = 6$) showing that peak height in PCPA incubated VNCs (triangles) decreases to $50 \pm 3\%$ of initial value by the 4th stimulation. Asterisks indicate data are significantly different (** $p < 0.01$, *** $p < 0.001$, student t-test, 2 tailed) than samples incubated in buffer (circles).

Fig. 5.

Pharmacological characterization of serotonin release and clearance. VNCs were incubated in drugs for 30 minutes prior to the initiation of stimulation. (a) Representative trace from a VNC incubated in the VMAT inhibitor reserpine, superimposed on a trace from a control VNC that lacks ChR2 expression showing that they are similar in size and shape. (b) Representative traces from VNCs incubated in buffer, cocaine, or fluoxetine, superimposed to highlight differences in time course. (c, d) Pooled data (mean $\pm$ SEM, n as shown). (c) Peak height is not affected by incubation in cocaine or fluoxetine (signal not different from buffer control group). Reserpine leads to a significant reduction in released serotonin ($p < 0.0001$, student t-test, 2 tailed), with the signal size falling below the noise threshold (signal not significantly different from control VNC). (d) Cocaine and fluoxetine-incubated VNCs exhibit significantly longer time to half maximal signal decay ($p < 0.0001$, student t-test, two-tailed). Clearance measures were not performed for control or reserpine-incubated VNCs because serotonin was not detected.

Fig. 6.

Kinetic parameters governing serotonin clearance. (a) Parameters can be calculated from the elicited serotonin peaks. The decay portion of the signal is fit with a one phase exponential decay curve. From this equation, k , the exponential constant is used to calculate an initial velocity (V). These parameters are shown (b, c) plotted against peak height (mean $\pm$ SEM of 3–12 signals per point) (b) The initial velocity of serotonin clearance data were fit to a 4 parameter logistics equation (line). (c) The derived clearance decay rate (k) decreases as larger serotonin release is elicited.