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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. Channelrhodopsin-2-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.

1. 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 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 Condron, 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 larva 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 central 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 larval fly 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

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utility of *Drosophila* as a model system for studying the basic mechanisms underlying neurotransmission.

2. Materials and methods

2.1. 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. (2003). For dopamine detection, the electrode potential was continuously scanned from −0.4 V 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 1000 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.

2.2. 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.

2.3. Preparation of ventral nerve cords

Five-day-old larval VNCs were dissected in modified Schneider's insect media (15.2 mM $MgSO_4$, 21 mM KCl, 3.3 mM KH_2PO4, 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 3 ml fresh buffer. The VNC was visualized under a 40× 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.

2.4. Data collection

Data collection was initiated no later than 50 min 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 Fig. 1). After the collection of at least 30 s of baseline data, VNCs were exposed to 10 s of intense blue light to stimulate release. The light source was a 10 W 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 min before the next stimulation. Peak height remained stable when 10 s 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 min before stimulation to allow time for drug diffusion.

2.5. Data analysis

Electrochemical data was analyzed using Tar Heel CV software (Heien et al., 2004). Data were visualized as a background-subtracted cyclic voltammogram, 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 s before blue light exposure. Signal CVs were collected approximately 1 s 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 100 s. Greater amounts of drift and larger peak shifts were observed when using the dopamine waveform. Statistical analysis of pooled data including two-tailed Student's t tests was conducted using Excel and InStat software. Curve fitting was done in GraphPad Prism.

3. Results

3.1. 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 *Chamydomonas reinhardtii* (Schroll et al., 2006). ChR2 allows neuronal excitation on a millisecond timescale and provides single action potential control of 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, respec-