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Neurochemistry **Hot Paper**

Fast-Scan Cyclic Voltammetry (FSCV) Reveals Behaviorally Evoked Dopamine Release by Sugar Feeding in the Adult *Drosophila* Mushroom Body

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Abstract: *Drosophila melanogaster*, the fruit fly, is an excellent model organism for studying dopaminergic mechanisms and simple behaviors, but methods to measure dopamine during behavior are needed. Here, we developed fast-scan cyclic voltammetry (FSCV) to track in vivo dopamine during sugar feeding. First, we employed acetylcholine stimulation to evaluate the feasibility of in vivo measurements in an awake fly. Next, we tested sugar feeding by placing sucrose solution near the fly proboscis. In the mushroom body medial tip, 1 pmol acetylcholine and sugar feeding released 0.49 ± 0.04 μM and 0.31 ± 0.06 μM dopamine, respectively but sugar-evoked release lasted longer than with acetylcholine. Administering the dopamine transporter inhibitor nisoxetine or D2 receptor antagonist flupentixol significantly increased sugar-evoked dopamine. This study develops FSCV to measure behaviorally evoked release in fly, enabling *Drosophila* studies of neurochemical control of reward, learning, and memory behaviors.

Dopamine signaling is important for mediating appetitive associative learning and memory both in mammals and insects.[1] *Drosophila melanogaster*, the fruit fly, is a powerful model system to understand how sensory inputs mediate behavioral responses because flies have robust appetitive olfactory learning and memory behavior.[2] In *Drosophila*, dopamine is a key neuromodulator for reward reinforcement, and sugar reward triggers the activation of distinct groups of dopaminergic neurons in the mushroom body (MB).[3] Recent studies in *Drosophila* revealed that a protocerebral anterior medial (PAM) dopaminergic neuron cluster, projecting to the MB γ lobe, is divided into 5 distinct compartments. The medial tip, the γ4 and γ5 compartments of the MB γ lobe, is activated during sugar reward to form appetitive memories.[4,5] These studies used behavior, immunostaining, and functional imaging to track which dopamine circuits control behavior. Electrophysiology reveals firing patterns of individual neurons but does not identify neurochemicals or quantify the amount of released.[6] Dopamine release is highly dynamic due to complex regulation by dopamine autoreceptors and transporters.[7] Therefore, direct measurements of dopamine concentration changes are necessary to understand behaviorally evoked dopamine release in *Drosophila.*

Direct, in vivo measurements of dopamine release in *Drosophila* are challenging due to the small size of its nervous system. Genetically encoded fluorescent sensors can be expressed in neurons to specifically track dopamine release but they cannot be used for long-term measurements due to photobleaching and cannot be calibrated to provide concentration information.[8,9] Galvanic redox potentiometry and fast-scanning potential-gated organic electrochemical transistors demonstrate highly selective, reliable in vivo measurement of neurochemicals but are larger so they are not suitable for a small model system, like *Drosophila.*[10,11] Fast-scan cyclic voltammetry (FSCV) is an electrochemical technique that measures sub-second concentration changes and provides fingerprint cyclic voltammograms to identify neurochemicals.[12] FSCV has been previously employed to measure neuromodulators in *Drosophila* larval ventral nerve cord (VNC) and in the adult central complex and MB.[13–15] Moreover, a similar approach was implemented to measure dopamine in the honey bee.[16] However, these studies were performed either in ex vivo preparations or during aversive stimulation. Also, dopamine was measured in the calyx or vertical lobes of the MB, but not in smaller specific compartments of other lobes of the MB, which are activated by different behaviors.[17] Thus, the goal of this study was to develop FSCV methods to measure dopamine in specific compartments in an awake, restrained fly during exogenous and behavioral stimulations. The significance of this work is that it demonstrates for the first time new neurochemical knowledge, that dopamine release to appetitive stimulation in specific compartments of the *Drosophila* mushroom body. The broader significance is that this shows that FSCV and CFMEs are good for probing reward-related neurotransmitter signaling and this will have broad implications for understanding how neurotransmission controls reward in a model system that is useful for genetic interrogation and disease models.

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Here, we developed in vivo FSCV to measure dopamine release in the MB medial tip during behavior. An anesthetized, adult fly was immobilized in a customized chamber and the cuticle of the dorsal fly head was surgically removed to expose the brain for carbon-fiber microelectrode (CFME) implantation (Figure 1A–E). The MB was visualized using a green fluorescent protein (GFP) marker by crossing dopamine transporter-galactose 4 (DAT-Gal4) flies with upstream activating sequence (UAS)-GFP flies. The CFME is 7 μm in diameter and 50 μm long; thus it provides sufficient spatial resolution to probe dopamine signaling in the MB medial tip (about 25 μm width and 40 μm length).[3] A capillary filled with 5 mM acetylcholine was implanted 10 to 15 μm away from the electrode. This preparation allows FSCV measurements while the fly is free to breath and move underneath the chamber for sugar intake.

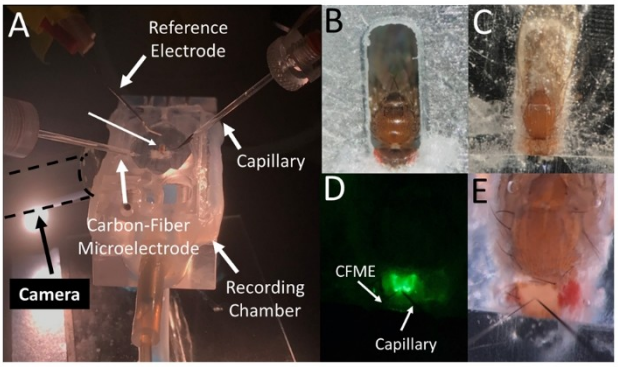

Figure 1. In vivo *Drosophila* brain preparation. A) Picture of setup of in vivo FSCV measurements in adult fly brain. B) An anesthetized fly was immobilized in a recording chamber. (C) The cuticle of dorsal head was removed to expose the brain. D) A fluorescence image of adult fly (DAT-Gal4;UAS-GFP) with GFP expressed in dopaminergic neurons in the MB. E) Electrode implantation. A CFME and a capillary filled with acetylcholine were positioned in the medial tip of the MB.

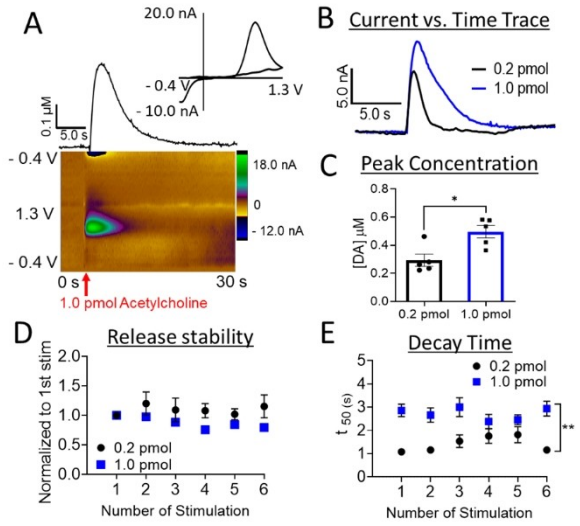

Figure 2. In vivo FSCV acetylcholine-stimulated dopamine in the MB medial tip of the adult fly. A) Example data of 1.0 pmol acetylcholine-induced dopamine release. Color plot (bottom) and cyclic voltammogram (inset) identified dopamine release. Comparisons of 0.2 and 1.0 pmol acetylcholine stimulation for B) current vs. time traces, C) average peak concentration, D) release stability, and E) half-decay time of released dopamine. $*p<0.05$, $**p<0.01$. Statistical analysis in Table S1.

First, we confirmed that dopamine could be measured in a restrained fly by doing acetylcholine stimulations in the MB medial tip. Acetylcholine stimulation does not require any genetic manipulation, such as optogenetics and chemogenetics.[13] In the fly, acetylcholine activates widely expressed endogenous nicotinic acetylcholine receptors causing fast dopamine release.[14] Figure 2A shows representative data of 1.0 pmol acetylcholine-evoked dopamine release. Dopamine concentration increases rapidly and returns to baseline in about 10 s, which is longer than in an ex vivo preparation.[13] The color plot and cyclic voltammogram confirm the measured signal is dopamine. We compared in vivo dopamine release evoked by either 0.2 or 1.0 pmol acetylcholine (Figure 2B). More dopamine and slower uptake were observed with the higher acetylcholine application. Current responses are converted to concentration using a calibration factor. The peak concentration was significantly higher with 1.0 pmol (Figure 2C, 0.49 ± 0.04 μM, $n=5$) acetylcholine stimulation than with 0.2 pmol (0.29 ± 0.06 μM, $p=0.1113$, $n=5$, full statistics in Table S1).

To investigate the stability of dopamine release, acetylcholine stimulations were repeated at 5 min intervals (Figure 2D,E). There are no significant effects of stimulation amount or stimulation number on dopamine concentration or the half-decay time (t_{50}), i.e. the time it takes to decrease from the maximum to its half concentration. However, 1.0 pmol acetylcholine (2.7 ± 0.1 s) had significantly slower clearance than 0.2 pmol stimulation (1.4 ± 0.1 s, $p=0.0020$). Acetylcholine-evoked dopamine release (0.49 ± 0.04 μM) in vivo was similar to that previously measured in an ex vivo preparation (0.38 ± 0.10 μM).[18] However, acetylcholine stimulated release was stable at 5 min intervals in vivo whereas a 10 min interval is required for stable release in ex vivo experiments. Enhanced stability could be due to a more rapid replenishment of the dopamine releasable pool in vivo. These acetylcholine results show that dopamine release can be measured in a restrained, but unanesthetized *Drosophila* brain.

To demonstrate in vivo FSCV during behavior, we measured dopamine in the MB medial tip during sugar feeding. Figure 3 shows the sugar feeding setup and in vivo dopamine release measured during a single trial of sugar feeding. The tip of the syringe, filled with sugar solution, was raised until the tip of fly's proboscis touched the sugar solution to initiate feeding and removed after 10 s (Figure 3A–C). To confirm sugar ingestion, the 1 M sucrose solution was mixed with red food coloring (mix of RED dye #3 and #40), and the red color is seen in the fly stomach after feeding. The concentration trace and color plot show current increases after sugar feeding (Figure 3D,E). The behaviorally evoked cyclic voltammogram is similar to from acetylcholine stimulation, identifying the main compound

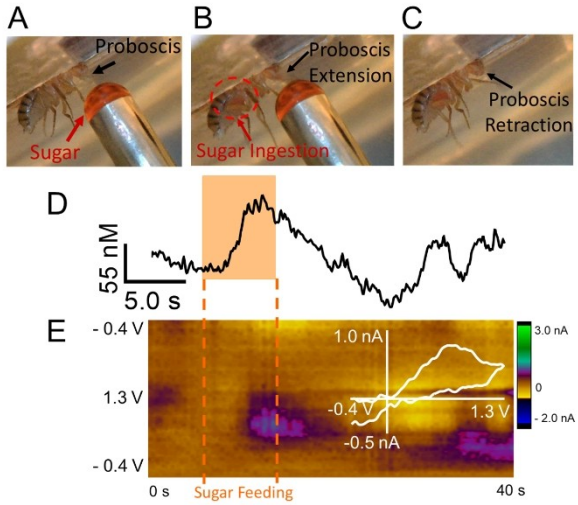

Figure 3. In vivo FSCV data recorded during a single trial of sugar feeding. Pictures of the fly A) before, B) during, and C) after sugar feeding. D) Dopamine release is associated with sugar feeding as the concentration increases during the sugar feeding. E) The color plot also shows current changes during sugar feeding and the measured signal is identified as dopamine by evaluating the cyclic voltammogram (inset).

released as dopamine (Figure 3E, inset). In this particular example, 0.11 μM dopamine is released but the rise time and decay of dopamine is longer compared to the acetylcholine stimulated responses. In *Drosophila*, a previous study showed that higher sugar concentration elicited stronger proboscis extension response,[19] and so future studies could test various concentrations of sugar to explore the association between sugar concentration and dopamine release.

One interesting observation is that dopamine rises slowly in response to the sugar feeding, and the response lasts for about 10–20 s. This delayed response could be due to diffusion of dopamine to the electrode, but the electrode was placed in the medial tip of MB, which is innervated with dopaminergic neurons associated with sugar reward. A similar phenomenon was observed in behavior studies in rats, where phasic dopamine changes were measured a few seconds after an unpredicted food reward (sugar pellet) was delivered.[7] Also, behaviorally evoked serotonin responses in the *Drosophila* MB calyx were delayed from the presentation of an odorant stimulant.[15] The delay could be caused by dopamine being indirectly activated by multiple modulatory pathways. Imaging and genetic studies revealed that octopamine triggers dopamine activity for sugar reward to form appetitive memory by regulating dopaminergic neurons in the MB.[20,21] Although octopamine release is not observed in the cyclic voltammogram, we could further explore octopamine-reinforcement on dopamine signaling by detecting both neurotransmitters either locally or distantly during sugar feeding. Further investigation could also evaluate other brain regions to determine if the delay is universal and probe dopamine signaling in specific dopaminergic clusters within the MB, like PAM neurons.

During the behavioral experiment, we repeated sugar delivery three times at 10 min intervals (top, Figure 4A). Release was similar for each feeding, showing that multiple measurements can be made in one animal. The baseline does fluctuate some in the in vivo traces, likely due to both electrode drift and also slow changes in electroactive compounds that contribute to the background current. However, it is stable enough to see the large changes due to sugar feeding. The fly cannot take more than three, 10 s sugar feedings since its abdomen gets too full. For future experiments, a shorter time of sugar feeding will be applied to increase the number of feeding sessions.

Dopamine signaling is mediated by DAT and dopamine 2-like (D2) receptors in mammals, that regulate dopamine concentrations in the extracellular space.[22] In the fly, *Drosophila* D2 receptors (DD2Rs) share the highest protein homology with mammalian D2 receptors,[23] and FSCV showed DD2Rs are autoreceptors that regulate dopamine in larva.[24] In the MB, DD2Rs are highly expressed and regulate appetitive long-term memory.[25,26] Flupentixol is a D2 antagonist used to treat schizophrenia and depression in humans.[27] Flupentixol is one of the few antagonists that showed activity toward DD2R receptors transfected in HEK293 cells, and flupentixol administration to third instar larvae caused abnormal melanization and cuticle defects.[28,29] *Drosophila* DAT (dDAT) has a similar functioning protein motif to mammalian DAT and modulates olfactory learning and memory formation.[30,31] Nisoxetine is a selective reuptake inhibitor for norepinephrine in mam-

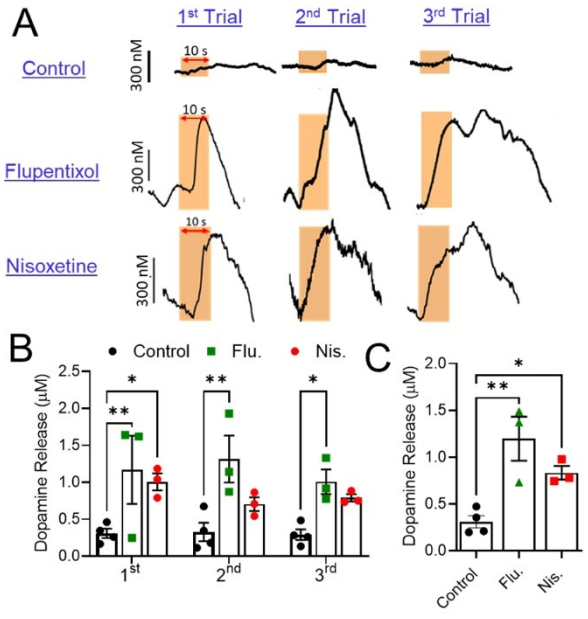

Figure 4. Behaviorally evoked dopamine release during control and after 20 μM D2 receptor antagonist flupentixol or DAT inhibitor nisoxetine. A) Example traces, B) average dopamine peak concentration by trial, and C) average dopamine concentration with the trials combined show significant dopamine increase in response to flupentixol and nisoxetine. $*p<0.05$, $**p<0.01$. Statistical analysis in Table S1.

mals, but in flies it is a selective dDAT inhibitor because *Drosophila* do not use norepinephrine.[30,32,33] Previously, flupentixol or nisoxetine increased stimulated dopamine in the *Drosophila* larval VNC.[33,34]

Here, we characterized drug effects by perfusing 20 μM flupentixol or 20 μM nisoxetine over the fly's head and measured sugar-evoked dopamine release. Because excess fat and air sacs were removed from head, the drugs can diffuse into the brain and 15 min is sufficient to observe the effect of drugs on acetylcholine-evoked dopamine signaling (Figure S1–S2). Figure 4A shows example FSCV traces of sugar-evoked dopamine release during the control (top, no drug), flupentixol (middle) or nisoxetine (bottom) perfusion. The concentration of dopamine release is higher after nisoxetine or flupentixol, indicating that DD2R and dDAT regulate its release. For repeated sugar feeding, each feeding evoked robust dopamine release with flupentixol or nisoxetine, similar to the control without drugs (Figure 4B).

Figure 4C displays average data, where dopamine release from 3 sugar stimulations in an individual fly was averaged. There is a significant effect of drug on the average dopamine release ($p = 0.0058$, $n = 3$–4). The average dopamine concentration is about 4 times higher than control with flupentixol and about 3 times higher with nisoxetine (Figure 4C, flupentixol 1.2 ± 0.3 μM, $n = 3$; nisoxetine, 0.83 ± 0.07 μM, $n = 3$; control, 0.31 ± 0.06 μM, $n = 4$). Similar results were observed when the drugs were applied and dopamine was stimulated by acetylcholine (Figure S1–S2). Nisoxetine also increased the half-time of sugar-evoked release (t_{50}) by about 2-fold (Figure S3). Therefore, dDAT and DD2Rs modulate dopamine release during sugar feeding.

In mammals, cue-mediated dopamine responses involve D2 receptors, and spontaneous dopamine transients are regulated by D2 receptors and DAT.[35,36] The regulation of dopamine dynamics by DD2R or DAT in adult flies is much less studied. Our measurements demonstrated DD2Rs and dDATs expressed in the MB medial tip regulate dopamine during sugar feeding behavior in the fly. Our results also show a wide dynamic range for behaviorally evoked dopamine release (nM to μM). DD2Rs here act as autoreceptors to presynaptically regulate dopamine release, and are a target that can be genetically or pharmacologically targeted to alter dopamine release. DAT is the main clearance mechanism and blocking it results in large extracellular dopamine concentrations and slower clearance of dopamine. To truly understand how dopamine regulates reward behaviors in the fly, it is important to probe dopamine signaling in each discrete MB compartment because different compartments regulates different functions, translating specific input signal into adaptive behavior responses. Our data shows compartment specific dopamine release and how the regulation of behaviorally evoked dopamine in flies is similar to mammals. Therefore, *Drosophila* is a good model system to understand D2 and DAT control dopamine in distinct MB compartments during reward.

In summary, we developed in vivo FSCV to probe endogenous dopamine release in the specific MB compartments in adult *Drosophila* brain. Acetylcholine stimulation validated the feasibility of in vivo FSCV detection in a restrained, living fly. FSCV was used for the first time to measure dopamine release during sugar feeding in the *Drosophila* MB medial tip. The magnitude of the sugar-evoked release was similar to acetylcholine, but the behaviorally evoked response was more sustained, having a slower rise time but also a slower clearance. Dopamine release was larger after applying flupentixol and nisoxetine, demonstrating D2 receptors and DAT mediate sugar-evoked dopamine signaling. This study provides new neurochemical information in a very useful model system that has not been used previously to study behaviorally evoked neurochemical changes. The novel perspective on real-time dopamine dynamic in specific MB compartments is critical to understanding how a specific set of dopaminergic neurons control behaviors. Additionally, real-time monitoring of dopamine in the MB medial tip lays a groundwork for studying other neurotransmitters in discrete MB compartments and understanding the anatomical difference in neurotransmitter signaling in behaviors, such as learning and memory formation.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: Dopamine Release · *Drosophila Melanogaster* · Fast-Scan Cyclic Voltammetry · Neurochemistry · Sugar Feeding

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