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Kinetics of the Dopamine Transporter in *Drosophila* Larva

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ABSTRACT: Dopamine transporters (DAT) regulate neurotransmission and are important in diseases such as addiction and attention deficit hyperactivity disorder. The *Drosophila* dopamine transporter (dDAT) is analogous to the mammalian DAT, but Michaelis–Menten kinetic parameters have not been characterized *in vivo*. In this study, dopamine clearance kinetics were measured in a *Drosophila* larval CNS using an implanted carbon-fiber microelectrode and fast-scan cyclic voltammetry. Dopamine was pressure ejected from a micropipet implanted 15–20 μm from the microelectrode. Clearance of exogenously applied dopamine was significantly reduced in dDAT null (*fumin*) mutants, and kinetic constants in these mutants were used to determine clearance by other mechanisms including diffusion. After correction for diffusion, the maximal rate of uptake, V_{max}, was estimated to be 0.11 ± 0.02 μM/s and K_m was 1.3 ± 0.6 μM in wild-type flies. The clearance rate was significantly reduced following treatment with the DAT inhibitor cocaine in wild-type flies, but not in *fumin* mutants, which indicates that serotonin transporter is not contributing significantly to dopamine clearance in these larvae. Clearance of endogenous dopamine, evoked by optical stimulation in flies expressing Channelrhodopsin2, was similar to clearance of exogenous dopamine, but it was not possible to evoke concentrations that were close to saturation. The ability to quickly assess the role of the dopamine transporter in any *Drosophila* larva will be useful for future studies of how transporters regulate neurotransmission and to understand the underlying mechanisms of drug addiction.

KEYWORDS: *Dopamine transporter, serotonin transporter, Drosophila, cocaine, fumin*

Neurotransmitter transporters are the key proteins that clear neurotransmitters from the extracellular space via uptake. Thus, the kinetics of these transporters regulate the amount of neurotransmitter available for signaling in the extracellular space. Transporters are often implicated in disease etiology and treatment. For example, most antidepressants target the serotonin or norepinephrine transporters while drugs of abuse and treatments for attention deficit hyperactivity disorder affect dopamine transporters (DAT).[1,2] Rapid screens of transporter kinetics in model organisms allow an understanding of how genetic mutations and pharmacological agents alter the extracellular concentrations of neurotransmitters.

In mammals, electrochemical sensors have been used to study the release and clearance of electroactive neurotransmitters in vivo and in brain slices.[3−5] There are two main electrochemical methods for studying transporter activity: measuring clearance rates of electrically stimulated release[6] or exogenously applied neurotransmitter.[7] With both methods, Michaelis–Menten kinetic constants for uptake can be estimated and the effects of pharmacological agents that inhibit the transporter measured. For example, the DAT inhibitor cocaine prolongs dopamine clearance and increases stereotypic behavior.[8] Dopamine clearance has also been studied in DAT knockout mice[9] and mice overexpressing DAT.[10] Mice lacking functional DAT exhibit prolonged dopamine signaling and are hyperlocomotive, while mice overexpressing DAT show increased rates of uptake and increased locomotor response to amphetamine.[1,8,11]

Genetically altered mice can take years to make, while *Drosophila* genetic models can often be produced in a few months. *Drosophila* DAT has a functional profile with characteristics resembling those of both mammalian norepinephrine transporters and DATs, and there is evidence implying the dDAT gene is a common ancestral gene for the vertebrate catecholamine transporters.[12] Both mammalian and *Drosophila* DAT have similar protein motifs and substrate selectivity.[12] Thus, rapid screening of the effect of genetic mutations on transporter function in *Drosophila* could serve as a basis for better understanding of the genetic components of mammalian transporter function. The Ewing group measured clearance of exogenously applied dopamine in the protocerebral anterior medial region of the adult *Drosophila* brain and found slower clearance after cocaine and in *fumin* mutants,[13] which lack a functional DAT.[14,15] Our lab pioneered direct measurements of endogenous dopamine and serotonin in *Drosophila* larva,[16−18] but kinetic constants such as K_m and V_{max} are difficult to determine because the amount of dopamine that can be evoked is limited.

In this paper, we use fast-scan cyclic voltammetry at carbon-fiber microelectrodes to measure Michaelis–Menten kinetic parameters of dDAT in intact *Drosophila* larval ventral nerve

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cords. The maximum rate of clearance, V_{max}, is of the same magnitude as the cortex and nucleus accumbens in mammals, and the affinity, K_m, is about 1 μM, similar to mammalian values. Clearance is slower in *fumin* mutants that lack DAT and in wild-type flies after application of cocaine, a transporter inhibitor. The ability to measure transporter kinetics in larvae will allow the function of transporters to be studied throughout *Drosophila* development and facilitate studies of uptake in disease models in *Drosophila*.

RESULTS AND DISCUSSION

Dopamine Uptake from Stimulated Release and via Application of Exogenous Dopamine. Dopamine-specific release can be optically evoked in *Drosophila* expressing Channelrhodopsin2 (ChR2) in dopaminergic neurons.[17] The duration of blue-light stimulation was varied from 3 to 12 s (Figure 1A). Maximal dopamine concentration reaches a plateau after about 7 s of blue light exposure, and the peak heights for 7 and 15 s stimulations are not significantly different (n = 5, paired t test, p = 0.86). The observed release plateaus with longer stimulations due to the depletion of the releasable dopamine pool and thus there is a balance between release and uptake.[19] The failure to return to baseline for the longer exposure to the blue-light may be due to a change in the background charging current caused either by ionic shifts in the tissue or a change of the electrode surface due to the light. In addition, carbon fiber microelectrodes do not always return to baseline when high concentrations are detected.[20]

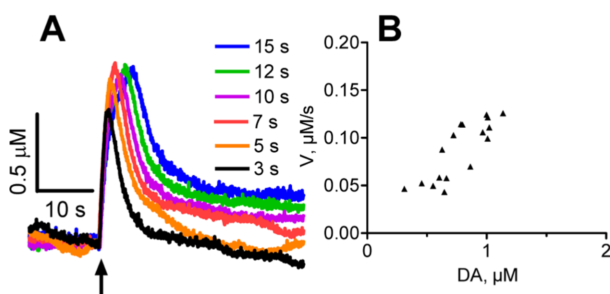

Figure 1. Effect of stimulation duration on dopamine signaling. (A) Stimulations of varying duration were applied by exposing the VNC to blue light. Examples of different length (3−15 s) stimulations are shown for one nerve cord demonstrate that the concentration detected does not rise past about 10 s of stimulation. (B) All stimulated release data were fit with an exponential decay and the initial velocity of clearance calculated. Initial velocity is plotted versus peak concentration detected for the stimulated dopamine release.

Figure 1B plots the clearance rate versus concentration detected for stimulated dopamine release. Concentrations over 1.5 μM are not achieved, and the data do not appear to reach saturation. Thus, exogenous application of dopamine was used in this study to characterize dopamine transporter kinetics.

Dopamine uptake was also measured after exogenous application of dopamine in a larval CNS. The ChR2 expressing flies were used for the exogenously applied experiments so that data could be compared to stimulated release.[17] The ChR2 expressing flies have uptake rates that are not significantly different from Canton S (k = 0.047 s^{-1}, p = 0.08), a standard wild-type strain, or w^{1118} flies (k = 0.049 s^{-1}, p = 0.19), the background strain for the *fumin* flies. A microelectrode and a capillary micropipet filled with dopamine were implanted into the neuropil approximately 15−20 μm apart (Figure 2A). Picoliter volumes of dopamine were pressure-ejected into the neuropil, and dopamine clearance observed electrochemically. Figure 2B shows the concentration versus time profile for pressure ejection of 210 pL of 25 μM dopamine into the neuropil. The cyclic voltammogram confirms that the change in current is due to dopamine (inset). Figure 2C shows the concentration versus time profiles for varying amounts of dopamine applied in a single CNS. As the amount applied increases, the maximal dopamine concentration, $[DA]_{max}$, detected at the electrode increases (Figure 2D).

The time course of decay from $[DA]_{max}$ can be used to characterize dopamine clearance by fitting it with a single exponential decay, $[DA](t) = [DA]_{max}\, e^{-kt}$ where k is the first order rate constant.[21] In Figure 2B, the first-order exponential fit (green dashed line) is overlaid on a dopamine clearance curve. The decay was fit from the time the stimulation ended until 80% of the signal decayed, similar to previous studies,[18,21] and R^2 values were over 0.98. The rate constant was used to calculate the initial velocity, V, of dopamine clearance using $V = k[DA]_{max}$. The initial velocity was plotted against the maximal dopamine concentration (Figure 3A). The concentration of dopamine in the pipet and the amount injected were varied to span a wide range of dopamine concentrations. A nonlinear regression analysis was performed to fit the data in Figure 3A to the Michaelis−Menten equation:

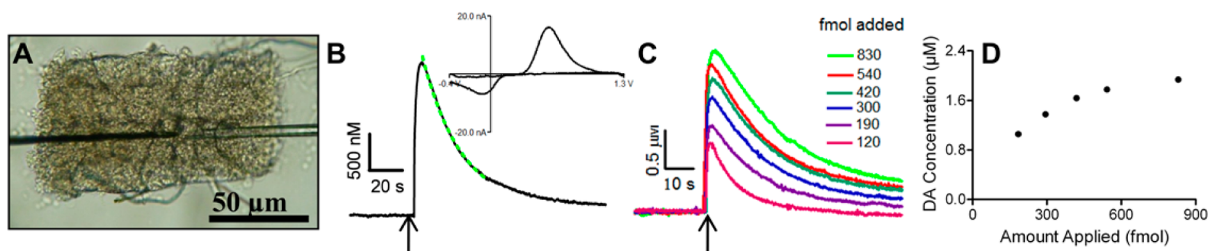

Figure 2. Application of exogenous dopamine in a *Drosophila* CNS. (A) An isolated *Drosophila* larval ventral nerve cord with both ends horizontally cut was adhered neuropil side down in a Petri dish. A carbon-fiber microelectrode and picospritzing capillary were simultaneously implanted approximately 15−20 μm apart in the neuropil. (B) Example concentration versus time trace detected at the electrode after 210 pL of a 25 μM dopamine solution was pressure ejected (arrow) into the tissue and dopamine detected. The inset CV confirms dopamine is detected. The green line is the exponential decay fit. (C) Representative traces of different amounts of exogenously applied dopamine in a single CNS. (D) In a single sample, the concentration of dopamine detected increases with the amount of dopamine applied, although the signal is not linear at higher amounts. Each point here is the peak concentration from the corresponding trace in panel (C).

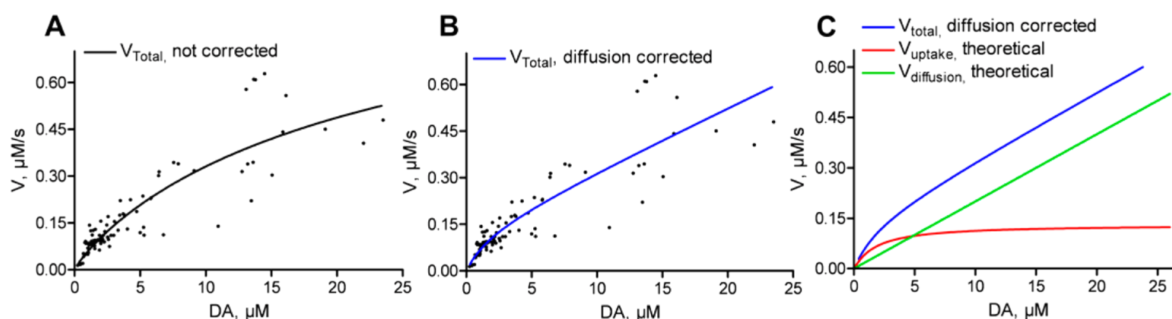

Figure 3. Initial velocity of clearance versus peak dopamine concentration detected. (A) All data (n = 93 traces from 29 animals) fit with Michaelis–Menten kinetics equation uncorrected for diffusion (black line). (B) All data fit with Michaelis–Menten kinetics equation that is corrected for diffusion (blue line). (C) Theoretical contributions of diffusion and uptake to clearance. The green line is the theoretical response for clearance velocity due only to diffusion and the red line is the theoretical response for only Michaelis–Menten uptake. These green and red lines add together to result in the blue line, with Michaelis–Menten kinetics corrected for diffusion.

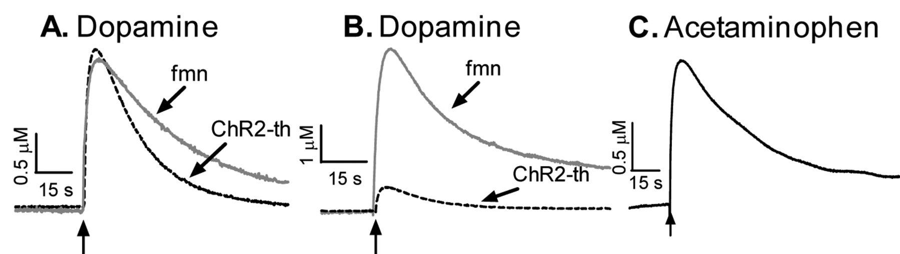

Figure 4. Application of exogenous dopamine in a *fmn* mutant. (A) Concentration versus time profile for a *fmn* larva (solid line, 0.5 pmol applied) compared to a UAS-ChR2;*th-GAL4* larva (dashed line, 5.3 pmol applied). For a similar concentration of dopamine detected, clearance in *fmn* is decreased. (B) Peak height is much larger in *fmn* compared to UAS-ChR2;*th-GAL4* when the same amount of dopamine is applied (1 pmol). (C) Application of 1 pmol of acetaminophen in a *fmn* fly indicates that the clearance for acetaminophen is similar to that of dopamine in *fmn* flies. Arrows indicate when the compound was applied.

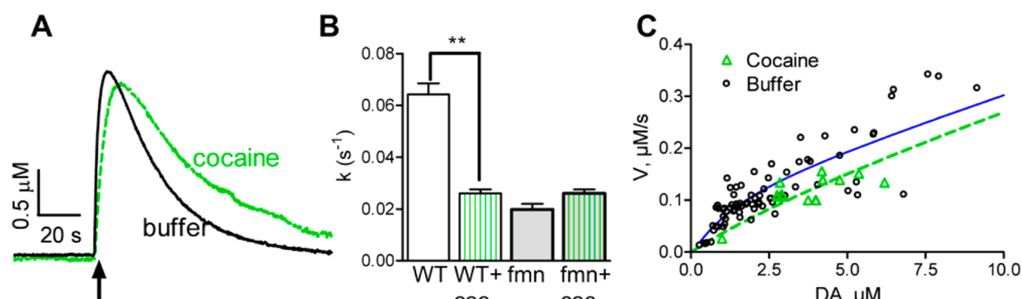

Figure 5. Application of exogenous dopamine in the presence of 50 μM cocaine. (A) Concentration versus time profile for the application of 1.1 pmol dopamine in a larva exposed to cocaine compared to 5.3 pmol of dopamine applied in buffer alone. For a similar concentration detected, clearance in the presence of cocaine is slowed. (B) The rate constant, k, of the WT (UAS-ChR2;*th-GAL4*) (n = 60) in buffer versus cocaine (n = 11) is significantly different after cocaine (unpaired t test, $^{**}p < 0.01$). The k for WT and cocaine is not different from that for *fmn* mutants, and the k for *fmn* is not significantly different after cocaine. (C) Initial velocity of clearance versus peak concentration plot comparing clearance in the presence of cocaine (green) versus buffer (black). The lines are the Michaelis–Menten fit corrected for diffusion. The observed affinity increases in the presence of a cocaine, a competitive inhibitor ($K_{m,obs}$ = 5.9 ± 1.0 μM).

$$V = \frac{V_{max}[DA]_{max}}{K_M + [DA]_{max}} \quad (1)$$

The Michaelis–Menten parameters are V_{max} = 0.98 ± 0.3 μM/s and K_m = 14 ± 10 μM (R^2 = 0.79, black line Figure 3A, n = 93 trials in 29 animals, error is SEM). However, these estimates do not take into account diffusion and other nonspecific clearance, which can distort K_m and V_{max} values,[22] so studies in DAT null flies were performed.

Dopamine Uptake in *fumin* Mutants. Exogenous application of dopamine provides a quick method to study transporter kinetics after genetic mutations. *Fumin* (*fmn*) mutants, which do not express DAT, have abnormally high levels of activity and reduced rest.[13] Makos et al. observed that clearance of applied dopamine in *fmn* adults was decreased.[14] *fmn* mutants are also a good model system to probe clearance mechanisms for dopamine without DAT, such as diffusion and nonspecific uptake.

Figure 4A shows the clearance of dopamine in a larval *fmn* mutant and a WT larva with a similar peak concentration of dopamine detected; however, only 0.5 pmol of dopamine was applied for *fmn* compared to 5.3 pmol for WT. Clearance is slower in *fmn*; however, dopamine is still cleared, indicating there are other clearance mechanisms besides DAT. When comparing two traces with similar amounts of dopamine applied (Figure 3B, 1 pmol applied), *fmn* mutants exhibit a 7-

fold larger peak height and a larger peak area. Similarly, in DAT knockout mice, 5-fold increases in extracellular concentration of dopamine have been reported.[9]

We fit decay curves from *fmn* (21 curves from 8 different animals) with a first-order decay function ($R^2 \geq 0.98$ for all). This allows the comparison of decay constants for the UAS-ChR2; *th-GAL4* larva ($k = 0.062 \pm 0.004$ s^{-1}) and the *fmn* larva ($k = 0.020 \pm 0.002$ s^{-1}). The decay was statistically different, indicating slower clearance in *fmn* larva (unpaired *t* test, $p < 0.001$). The *k* for w^{1118} flies, the background strain for *fmn* larva, was not different from that for UAS-ChR2;*th-GAL4* larva. A single exponential decay has been used to estimate the rate constant of dopamine clearance in DAT KO mice.[23] The *k* values for the *fmn* flies are similar to those in DAT knockout mice (0.038 s^{-1} nucleus accumbens shell and 0.032 s^{-1} nucleus accumbens core).[24]

Acetaminophen was used to track diffusion in *fmn* flies, as it is not a substrate for uptake or dopamine metabolic enzymes.[25,26] Acetaminophen is electroactive and can be monitored using carbon-fiber microelectrodes.[26] Figure 4C shows an example trace for acetaminophen clearance in a *fmn* fly. The average decay rate for acetaminophen was $k = 0.018 \pm 0.005$ s^{-1}, which is not different from dopamine (*t* test, $p = 0.90$, $n = 21$ for dopamine, $n = 14$ for acetaminophen). Thus, the clearance for dopamine in *fmn* flies appears to be due primarily to diffusion.

Dopamine Uptake in the Presence of Cocaine. Cocaine inhibits dDAT, significantly prolonging evoked dopaminergic signaling in *Drosophila* larva[17] and slowing clearance of exogenous dopamine in *Drosophila* adults.[15] Larval CNS were incubated in 50 μM cocaine for 15 min before application of exogenous dopamine.[16,27] Figure 5A compares clearance of 5.3 pmol of dopamine in a WT fly versus 1.1 pmol of dopamine after cocaine. The peak heights are similar, but the clearance is slower after cocaine. The average *k* after cocaine is 0.027 ± 0.002s $^{-1}$ ($n = 11$), which is significantly slower than that for buffer ($k = 0.060$ s^{-1}, $n = 60$) (Figure 5B, unpaired *t* test, $p < 0.01$). The clearance rate after 50 μM cocaine in WT flies was similar to that in *fmn* mutants, indicating that the majority of transporters had been blocked by this dose. For similar amounts of dopamine applied (data not shown), the dopamine currents are larger in cocaine than in buffer, similar to the results in *fmn* DAT null flies (Figure 4B). Increases in peak height following dopamine transporter inhibition have been reported in mammalian brain slice experiments[23,28] and *Drosophila* larvae.[17]

Cocaine has a higher affinity for the *Drosophila* serotonin transporter (dSERT) than for dDAT (464 and 2660 nM, respectively).[12] The serotonin transporter (SERT) can transport dopamine in *Drosophila*[12] and mammals.[29,30] To assess the extent to which dSERT might be involved in nonspecifc uptake, dopamine clearance was measured in a *fmn* mutant before and after application of 50 μM cocaine. Cocaine did not significantly alter the rate constant in *fmn* (Figure 5B, $n = 3$, paired *t* test, $p = 0.2127$), and the rate constants after cocaine are not significantly different for UAS-ChR2;*th-GAL4* ($n = 9$) and *fmn* ($n = 21$) (Figure 5B, unpaired *t* test, $p = 0.0869$). These results indicate that, in L3W *Drosophila* larval ventral nerve cords, dSERT is not contributing significantly to dopamine uptake. Future studies with adult fly brain and dSERT null mutants could enhance our understanding of the effect of dSERT on dopamine clearance.

Correcting Data for Non-dDAT Clearance. Dopamine clearance in *fumin* mutants that lack DAT was about 3 times slower than that in wild-type flies, indicating that about two-thirds of the clearance is due to DAT. However, *fmn* mutants do clear dopamine so other mechanisms of clearance must be corrected for. The experiments with *fmn* and cocaine indicated dSERT plays little role here in the clearance of dopamine. While the time scale for dopamine metabolism is not as well-defined in *Drosophila*,[31] the time-scale for dopamine metabolism in mammals is too slow (minutes)[32] to affect measurements of uptake.[33] The slow clearance of dopamine and acetaminophen in *fmn* mutants is similar and demonstrates that non-DAT clearance is primarily due to diffusion. Diffusion has been extensively studied in mammalian brains,[34,35] but measurements of tissue density, tortuosity, and the volume of extracellular space are unknown in *Drosophila*. Thus, we chose to use a simple exponential decay model to account for clearance not due to dDAT.

The *k* value from *fmn* mutants was used to correct V_{max} and K_m values for nonspecific clearance using this equation:

$$V = \frac{V_{max}[DA]_{max}}{K_M + [DA]_{max}} + 0.020[DA]_{max} \quad (2)$$

When corrected, $V_{max} = 0.11 \pm 0.02$ μM/s and $K_m = 1.3 \pm 0.6$ μM (blue line, Figure 3B, $R^2 = 0.78$). Figure 3C shows the corrected total curve (blue) as well as the theoretical curves for diffusion only (green) and Michaelis–Menten kinetics (red). As would be expected for a saturable uptake process, uptake is the dominant mechanism at lower concentrations and diffusion is most important at higher concentrations. Therefore, only concentrations less than 5 μM were used when computing average *k* values, to diminish effects of diffusion.

Previous studies have reported high values of K_m were experimentally determined as a result of diffusional processes coupled to uptake kinetics.[22] In the present study, V_{max} and K_m values were lower after correction for diffusion. V_{max} depends on the density of transporter expression, which can vary due to preparation or brain region. In transfected cells expressing dDAT, V_{max} is an order of magnitude larger (1.4 μM/s)[12] than the value we reported here, but the density of transporters in that preparation is likely different than in intact *Drosophila* tissue. In mammals, V_{max} is the highest in the striatum (0.2–4 μM/s, depending on the method of measurement)[21,36] but lower in other regions including the nucleus accumbens (0.09–2 μM/s), prelimbic cortex (0.12 μM/s), and cingulate cortex (0.06 μM/s).[37] Our corrected value for V_{max} in *Drosophila* larva (0.11 μM/s) is similar to that in mammalian brain regions with more modest DAT expression. The corrected K_m value for *Drosophila* larvae, 1.3 ± 0.6 μM, is the same order of magnitude as dDAT activity in transfected cells ($K_m = 4.8 \pm 0.4$ μM),[12] human DAT (hDAT) in transfected cells,[12] and in mammalian studies.[21,38,39] The similar values for K_m for hDAT and dDAT suggest studies of dopamine dysregulation in a *Drosophila* model will be relevant for human diseases.

To estimate the apparent K_m in the presence of cocaine, data were fit with eq 2 and V_{max} was constrained to 0.11 μM/s, because cocaine is a competitive inhibitor (Figure 5C).[40] The observed affinity, $K_{m,obs}$, of DAT with cocaine is 5.9 ± 1.0 μM, a 4.5-fold increase. The dose of cocaine (50 μM) was chosen to be an order of magnitude greater than previous reports of K_i (2.6 μM), but the amount that diffuses into the nerve cord tissue is not known so a true K_i cannot be calculated.

CONCLUSIONS

We have shown that fast-scan cyclic voltammetry can be used to measure clearance of exogenously applied dopamine by the *Drosophila* dopamine transporter. This method allows the first estimates of V_{max} and K_m in an intact *Drosophila* CNS. The measured transporter affinity is consistent with previously reported values of dDAT in transfected cells, and is similar to human DAT values. The similarity of real-time dopaminergic signaling in *Drosophila* and mammals validates *Drosophila* as a model system for studying DAT. Future studies using this method as a rapid screen of genetic mutations could help identify the genetic elements that are critical for DAT regulation of dopamine, and will lead to an increased understanding of dopamine homeostasis.

METHODS

Chemicals. Chemicals were purchased by Sigma-Aldrich (St. Louis, MO) and used as received unless otherwise specified. Solutions were made using Milli-Q water (Millipore, Billerica, MA). All electrode calibrations, drug solutions, and *Drosophila* preparations were made using a modified Schneider's buffer (15.2 mM $MgSO_4$, 21 mM KCl, 3.3 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). Larval food for stimulated release experiments was prepared with 10 mM all-trans retinal mixed with yeast and water.

Electrochemical Measurements. T-650 carbon-fiber microelectrodes were constructed as previously described.[17] A Dagan Chem-Clamp potentiostat (Dagan, Minneapolis, MN; custom modified) and Tar Heel CV software (gift of Mark Wightman, University of North Carolina) connected to a homemade breakout box were used to collect data. The electrode was scanned from −0.4 to 1.3 V and back at a scan rate of 400 V/s at 10 Hz. An Ag/AgCl reference electrode was placed in the Petri dish near the ventral nerve cord. Electrodes were calibrated with a 1.0 μM dopamine solution before and after use in situ. For cocaine experiments, a second calibration was performed in the presence of drug to account for possible drug effects on the electrode sensitivity.

Picospritzing capillaries were made by pulling a 1.2 mm × 0.68 mm glass capillary (A-M Systems, Carlsburg, WA) with a vertical pipet puller (Narishige, Japan). The capillary surface was beveled (Sutter Instrument Co., Novoto, CA) at an angle of 30°. Capillaries were filled with dopamine solutions ranging from 10 to 100 μM, and a Picospritzer III instrument used for pressure ejection (Parker Hannifin, Fairfield, NJ). Each pipet was calibrated by ejecting dopamine solution into oil, measuring the radius of the ejected droplet, and calculating the volume (volume = $4/3\pi r^3$).

Preparation of the *Drosophila* CNS. Flies containing UAS-ChR2 (Bloomington Stock Center, Bloomington, IN) were crossed to flies expressing th-GAL4 (a gift from Jay Hirsh, University of Virginia) to generate homozygous lines with a th-GAL4;UAS-ChR2 genotype. *Fumin* flies were a gift from Jay Hirsh. For stimulated release experiments, 3-day-old L3W larvae were allowed to feed on a mixture of all-trans retinal mixture in the dark for 2 days prior to experimentation. For all experiments, the CNS from a 5-day-old L3W larvae was dissected out in a modified Schneider's buffer and the optic lobes removed by making a horizontal cut in the anterior-most portion of the ventral nerve cord. For picospritzing experiments, an additional horizontal cut was made at the posterior-most portion of the ventral nerve cord to facilitate micropipet insertion. Isolated ventral nerve cords were adhered neuropil side down in a Petri dish with 3 mL of buffer. An electrode was inserted with a micromanipulator into the neuropil using the 40× water immersion lens on an Axio Examiner microscope (Carl Zeiss, Thornwood, New York) 4−6 segments away from the cut edge. For picospritzing experiments, the picospritzing capillary was inserted 15−20 μm away from the electrode. The electrode and capillary were allowed to equilibrate after implantation for 5 min prior to data collection. For drug experiments, 1 mL of 200 μM cocaine was added to the Petri dish containing 3 mL of buffer to make a final concentration of 50 μM. The nerve cords were incubated in cocaine for 15 min before applying dopamine.

Data Analysis. Data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA). Data were considered different at a 95% confidence level. Error bars are standard error of the mean. Curve-fitting of clearance data and nonlinear regression analysis was performed with GraphPad and the parameters are shown as mean ± SEM at a 95% confidence level.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

DAT, dopamine transporter; dDAT, *Drosophila* dopamine transporter; *fmn*, *fumin* mutant; L3W, wandering third instar larva; ChR2, Channelrhodopsin2; WT, wild type

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