Using *in Vivo* Electrochemistry To Study the Physiological Effects of Cocaine and Other Stimulants on the *Drosophila melanogaster* Dopamine Transporter

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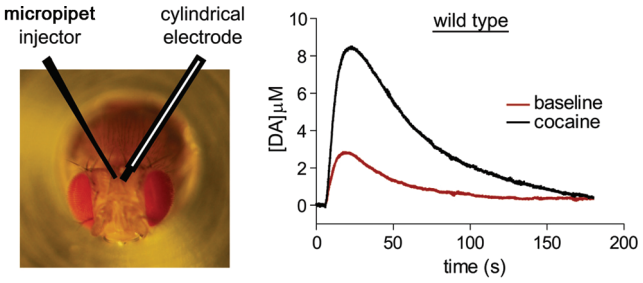
Abstract



Dopamine neurotransmission is thought to play a critical role in addiction, reinforcing mechanisms of drugs of abuse. Electrochemical techniques have been employed extensively for monitoring *in vivo* dopamine changes in the brains of model organisms including rats, mice, and primates. Here, we investigated the effects of several stimulants on dopamine clearance using recently developed microanalytical tools for *in vivo* electrochemical measurements of dopamine in the central nervous system of *Drosophila melanogaster*. A cylindrical carbon-fiber microelectrode was placed in the protocerebral anterior medial region of the *Drosophila* brain (an area dense with dopamine neurons), while a micropipet injector was positioned to exogenously apply dopamine. Background-subtracted fast-scan cyclic voltammetry was carried out to quantify changes in dopamine concentration in the adult fly brain. Clearance of exogenously applied dopamine was significantly decreased in the protocerebral anterior medial area of the wild-type fly following treatment with cocaine, amphetamine, methamphetamine, or methylphenidate. In contrast, dopamine uptake remained unchanged when identical treatments were employed in *fumin* mutant flies that lack functional dopamine transporters. Our *in vivo* results support *in vitro* binding affinity studies predicting that these four stimulants effectively block normal *Drosophila* dopamine transporter function. Furthermore, we found 10 μM to be a sufficient physiological cocaine concentration to significantly alter dopamine transporter uptake in the *Drosophila* central nervous system. Taken together, these data indicate dopamine uptake in the *Drosophila* brain is decreased by psychostimulants as observed in mammals. This validates the use of *Drosophila* as a model system for future studies into the cellular and molecular mechanisms underlying drug addiction in humans.

The psychomotor stimulant drugs cocaine, amphetamine, and methylphenidate all bind to the dopamine transporter and alter its function, increasing extracellular dopamine levels in the brain. The dopamine transporter is the plasma membrane protein primarily responsible for clearing dopamine from the extracellular space, which leads to the termination of dopamine neurotransmission (*1*, *2*). Several lines of evidence have demonstrated that increased extracellular dopamine levels underlie the reinforcing and addictive properties exhibited by drugs of abuse (*3*, *4*). It is well established that cocaine blocks dopamine uptake via the dopamine transporter to elevate the extracellular dopamine concentration (*5*, *6*), and more recently it has been thought to affect the serotonin and norepinephrine transporters as well (*7*, *8*). Amphetamine has dual effects on dopamine transport activity, both inhibiting dopamine uptake and inducing reverse transport through the dopamine transporter (*9*−*11*). Methylphenidate, a commonly prescribed medication for the treatment of attention deficit hyperactivity disorder (*12*), blocks the dopamine transporter and increases the synaptic dopamine concentration (*13*, *14*). While methylphenidate is abused by humans and has a similar affinity for the dopamine transporter as cocaine (*6*), abuse is not as widespread as that of cocaine. The pharmacokinetics of the two drugs is thought to contribute to the difference observed in their addictive properties (*15*). Neurochemicals in the central nervous system (CNS) associated with addiction have been investigated for several decades; however, the mechanisms underlying stimulant addictions and the behaviors they elicit are still not fully understood.

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Animal model systems including rats, mice, and primates have been used for several decades to study the effects of psychostimulants on dopamine transporter function (*3*, *7*, *16*, *17*). Recently, there is accumulating evidence for the validity of using *Drosophila melanogaster* as a model system for neurotransmission (*18*, *19*). The monoamines dopamine and serotonin play significant roles in regulating diverse physiological processes including attention, motivation, and addiction in humans and have been found to exert similar functions in the fly (*20*−*23*). When exposed to cocaine, nicotine, or ethanol, *Drosophila* exhibits behavioral responses akin to those displayed by mammals (*24*−*28*). In addition to the above-mentioned monoamines, octopamine is a major neurotransmitter in the CNS of invertebrates. Similar to norepinephrine in mammals, octopamine dynamics in *Drosophila* are affected by exposure to cocaine (*29*). Although behavioral studies are a crucial aspect of investigating psychostimulant actions, *in vivo* quantification of neurochemicals would greatly improve understanding of molecular and cellular pathways behind the reinforcing and addictive effects of a drug.

The electroactive nature of several neurotransmitters makes *in vivo* electrochemistry an ideal approach for measuring chemical changes in the brain. Uptake studies on both exogenously applied dopamine and stimulated dopamine release have been characterized *in vivo* using voltammetry and chronoamperometry techniques (*30*−*32*). Fast-scan cyclic voltammetry (FSCV) coupled with carbon-fiber microelectrodes is a valuable method for quantification of biogenic amines in the CNS because of its chemical selectivity and subsecond temporal resolution (*33*−*35*), and it has been used in rats, mice, *Drosophila* flies, and *Drosophila* larvae (*5*, *36*−*38*). Here, we utilized recently developed microanalytical techniques to measure changes in the uptake of exogenously applied dopamine in the CNS of adult *Drosophila* with treatments of cocaine, amphetamine, methamphetamine, or methylphenidate. The physiological stimulant concentration necessary to significantly block uptake by the dopamine transporter was approximately 10 μM.

Results and Discussion

Dopamine Uptake in Wild-Type vs *fmn* Mutant Flies Following 1.0 mM Cocaine Treatment

Microanalytical techniques developed for *in vivo* electrochemical detection in *Drosophila* provide a method for studying the physiological effects of drug treatments on redox-active neurotransmitters. Previously, we have characterized exogenously applied dopamine uptake using electrochemical detection with a carbon-fiber microelectrode inserted into the protocerebral anterior medial (PAM) area of an adult *Drosophila* brain (*38*). In this study, we utilized this procedure to explore dopamine neurotransmission in the *Drosophila* CNS. Dopamine neuronal cell bodies are clustered together in several distinct areas throughout the *Drosophila* brain with the largest neuronal cluster located in the PAM region projecting to the nearby mushroom body (*39*−*41*), a key brain structure for learning and memory (*42*). Octopamine levels in this particular brain region are insignificant, simplifying measurements of dopamine. Thus, we focused our *in vivo* investigation of dopamine uptake in *Drosophila* on the PAM area.

Following microsurgery, a micromanipulator was used to insert the cylindrical working electrode into the PAM region while the reference electrode was submerged in the AHL saline bath covering the exposed fly brain. Small amounts of dopamine were ejected just above the PAM area, approximately 10 μm from the working electrode, with a single micropipet injector. FSCV was used to monitor changing dopamine levels in the CNS of both wild-type and *fmn* mutants over time. Voltammetry was performed by applying potential in a triangular waveform (scanning from −0.6 V to +1.0 V then back to −0.6 V vs a Ag/AgCl reference electrode) to the electrode, while the current response was recorded. This waveform was applied at 200 V/s every 100 ms throughout the length of an experiment. To visualize changes over time, a false-color representation of current is used (Figure 1A) where the green corresponds to the oxidation of dopamine, and the reduction of the orthoquinone is represented in blue (*33*). The current response was converted to dopamine concentration using *in vitro* electrode calibration. The peak dopamine concentration measured is referred to as $[DA]_{max}$, which is an established parameter for measuring changes in uptake of extracellular dopamine (*17*). In addition to $[DA]_{max}$, another parameter used to compare dopamine clearance between the two fly genotypes is $t_{1/2}$, the full width of time at half-maximum of the dopamine concentration (Figure 1B).

The validity of using $[DA]_{max}$ to compare changes in dopamine uptake via the functional dopamine transporter in wild-type flies vs the nonfunctional dopamine transporter in *fmn* flies has been demonstrated (*38*). Here, $[DA]_{max}$ was used to investigate the effectiveness of a known dopamine uptake inhibitor, cocaine, on blocking uptake by the *Drosophila* dopamine transporter *in vivo*. A 1.0 mM dopamine solution was exogenously applied to the PAM area for 1.0 s (corresponding to ∼150 pmol of dopamine ejected), and the current response was recorded for 3 min (Figure 2A,B, "baseline 1"). Following three baseline measurements, the fly brain was bathed with 1.0 mM cocaine in AHL saline for 5 min, and then the current response was recorded over time following dopamine injection

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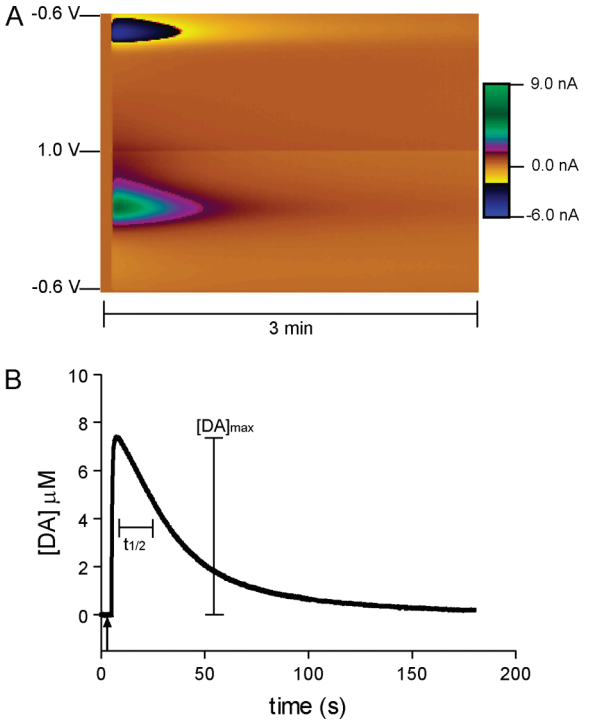

Figure 1. *In vivo* detection of exogenously applied 1.0 mM dopamine in the adult *Drosophila* brain: (A) applied potential vs time gives a visual representation of successive voltammograms with current viewed in false color; (B) dopamine concentration plotted over time. Dopamine concentration was determined from the measured current using an *in vitro* calibration average of three electrodes. The black arrow corresponds to a 1.0 s dopamine application beginning at 5.0 s.

("5 min cocaine"). Cocaine treatment was continued and dopamine injections were repeated every 5 min while the current response was recorded.

The representative cyclic voltammogram in Figure 2C is a background-subtracted average of ten successive cyclic voltammograms acquired during an *in vivo* dopamine baseline measurement from an adult wild-type fly brain (dashed red line). A background-subtracted average of ten successive cyclic voltammograms of exogenously applied dopamine following 15 min of 1.0 mM cocaine treatment is plotted for comparison (solid black line). Both voltammograms are from the time period when $[DA]_{max}$ was measured, and by inspection, the voltammetric peaks correspond to the electrochemical signature of dopamine (*35*, *43*). After a 1.0 mM cocaine treatment, a 3-fold increase in $[DA]_{max}$ was observed for the adult wild-type fly (Figure 2A), while the $[DA]_{max}$ of the *fmn* mutant fly (Figure 2B) remained unchanged. Notably, comparison of the baseline measurements in Figure 2A,B shows a significant difference between the two fly types following exogenous dopamine application. Less dopamine is detected in the wild-type flies vs the *fmn* flies, which is likely due to dopamine uptake by the functional transporter, which is

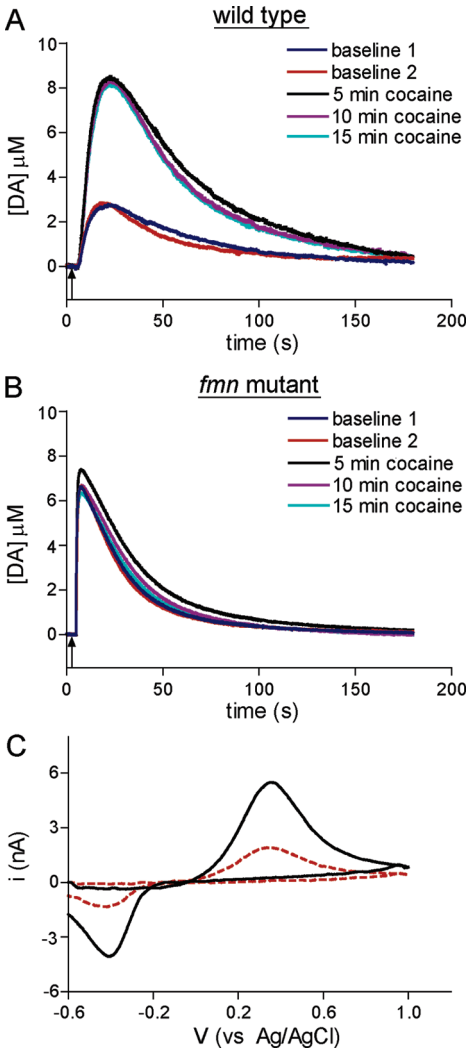

Figure 2. Effect of 1.0 mM cocaine treatment on uptake of an exogenously applied 1.0 mM dopamine solution: (A) Representative concentration trace of exogenously applied dopamine in the wild-type fly before (baseline 1, 2) and after 1.0 mM cocaine treatment. A significant increase in dopamine concentration was observed. (B) Representative concentration trace of exogenously applied dopamine in the *fmn* mutant fly before (baseline 1, 2) and after cocaine treatment. No significant change was observed for the *fmn* mutant fly. Dopamine concentration was determined by converting the measured current using *in vitro* electrode calibration. The black arrow corresponds to a 1.0 s dopamine application beginning at 5.0 s. (C) Background-subtracted fast-scan cyclic voltammogram of baseline dopamine (dashed red line) compared with dopamine after 15 min of 1.0 mM cocaine treatment (solid black line) in the wild-type fly (average of 10 scans).

present only in the wild-type flies. This observation has been reported in detail previously (*38*). Upon comparison of the two genotypes, wild-type flies exhibited a significantly increased normalized $[DA]_{max}$ with 1.0 mM cocaine treatment compared with *fmn* mutant flies with the same treatment (Figure 3; two-way analysis of variance (ANOVA); $p = 0.0002$ for interaction, $n = 6$). To account for slight differences in dopamine injector

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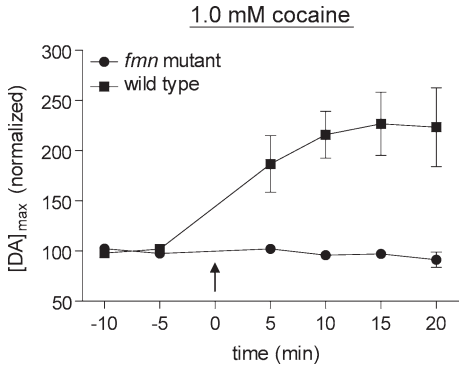

Figure 3. Comparison of wild-type and *fmn* mutant flies when 1.0 mM dopamine was exogenously applied before and after 1.0 mM cocaine treatment. There is a significant increase in normalized $[DA]_{max}$ for wild-type flies vs *fmn* flies with cocaine treatment (mean ± SEM; two-way ANOVA; $p = 0.0002$ for interaction, $n = 6$). The black arrow corresponds to the beginning of the 1.0 mM cocaine treatment.

positioning between flies, the $[DA]_{max}$ from two of the dopamine baseline measurements for a fly were averaged together, and all measurements for that fly were calculated as a percent of the average baseline measurement (i.e., $[DA]_{max}$ normalized) (*38*, *44*, *45*). The maximum effect of the cocaine treatment on the wild-type flies was observed within 10 min and remained fairly constant for over 20 min of cocaine treatment, while neither genotype experienced a significant change in $t_{1/2}$. These observations indicate that cocaine effectively blocks the *Drosophila* dopamine transporter function *in vivo*.

Determination of the Physiological Cocaine Concentration in the *Drosophila* Brain Region

To estimate the concentration of the 1.0 mM cocaine solution in the PAM area, APAP was used to mimic the bath application method of the cocaine treatment. APAP was selected because it is an electroactive molecule that is thought to undergo neither rapid metabolism nor uptake by monoamine transporters, thus allowing only the oxidation current from diffusion of the 1.0 mM bath solution into the brain region to be measured (*46*). Furthermore, detection of APAP using voltammetry is well documented (*47*, *48*). To determine the physiological drug concentration in the *Drosophila* brain region from a 1.0 mM bath application over the experimental time period, a carbon-fiber microelectrode was placed in the PAM region of *Drosophila*, and the fly head was bathed in 1.0 mM APAP in AHL saline solution. Background-subtracted FSCV was performed to measure the current *in vivo* from oxidation of APAP at the surface of the implanted electrode (Supplemental Figure 1A, Supporting Information). The peak oxidation current was converted to APAP concentration, [APAP], using *in vitro* electrode calibration with APAP (Supplemental Figure 1B, Supporting Information). The actual [APAP] in the *Drosophila* brain, or the physiological [APAP], is approximately 2 orders of magnitude lower (12 ± 5 μM, $n = 3$ flies) than the applied 1.0 mM bath [APAP]. While the concentration that diffuses into the tissue might differ slightly between cocaine and APAP due to the distinct properties of the two species, such as diffusion rate and relative permeability into the tissue, this difference is insignificant compared with the high resistance to diffusion of the brain tissue. When these calculations are applied to the cocaine solutions, a 1.0 mM cocaine bath application corresponds to approximately a 12 μM or 0.004 mg/mL cocaine concentration in the PAM area. This is significantly lower than that used in a study by Hirsh and colleagues where 0.5 mg/mL cocaine was applied directly to *Drosophila* nerve cords (*20*). Interestingly, our physiological cocaine concentration is consistent with a recent report by Venton and co-workers, which found that 10 μM cocaine was sufficient to effectively block serotonin reuptake by serotonin transporters located in the ventral nerve cords of *Drosophila* larvae (*37*).

Drosophila Dopamine Transporter Inhibition as a Function of Cocaine Concentration

Electrochemical detection with FSCV was used to investigate the effect of three different concentrations of cocaine (0.05, 0.5, or 1.0 mM) on dopamine uptake by the *Drosophila* dopamine transporter. The fly was prepared for *in vivo* electrochemical measurements (*vide supra*) and bathed with 0.05 mM cocaine in AHL saline after the baseline dopamine measurements were acquired. Voltammograms of 1.0 mM dopamine injections were obtained every 5 min. Figure 4 is a comparison of the normalized $[DA]_{max}$ for wild-type vs *fmn* mutant flies after separate treatments for 10 min with 0.05, 0.5, or 1.0 mM cocaine. We used a two-way ANOVA to analyze the comprehensive data at all doses and a significant difference in normalized $[DA]_{max}$ was observed between the two fly genotypes and the cocaine concentration (two-way ANOVA; $p < 0.0001$ for genotype, concentration, and interaction, $n = 6$ for each concentration and genotype). In addition, wild-type flies incubated with 1.0 mM cocaine had significantly increased normalized $[DA]_{max}$ compared with control measurements of AHL saline only (one-way ANOVA; $p < 0.0001$, followed by *post hoc* Tukey pairwise comparisons; $p < 0.0001$, $n = 6$). Higher dopamine concentrations were detected in wild-type flies treated with 0.5 mM cocaine as well; however, the effect was not as robust as that observed with the 1.0 mM cocaine treatment ($[DA]_{max}$ increased ~20% vs ~125% compared with AHL treatments). When the applied cocaine concentration was further decreased to 0.05 mM, there was no significant difference in the normalized $[DA]_{max}$ for wild-type flies from AHL saline measurements. Neither

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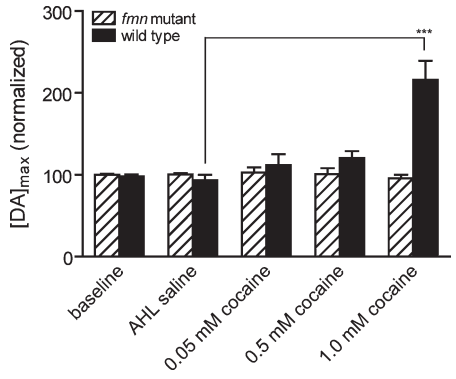

Figure 4. Comparison of wild-type and *fmn* mutant flies when 1.0 mM dopamine was exogenously applied before (baseline) and after 10 min of one of the following treatments: AHL saline only or 0.05, 0.5, or 1.0 mM cocaine solution (mean ± SEM; two-way ANOVA; $p < 0.0001$ for genotype, concentration, and interaction, $n = 6$). The bath solutions for the baseline and AHL saline treatment were identical. The AHL saline treatment was a control to ensure the $[DA]_{max}$ response did not increase from a temporal effect owing to the control solution. There is a significant increase in normalized $[DA]_{max}$ for wild-type flies after cocaine treatments compared with AHL saline (no cocaine) treatment (one-way ANOVA; $p < 0.0001$, *post hoc* Tukey pairwise comparisons; $p < 0.0001$ (***) for the 1.0 mM cocaine treatment, $n = 6$; SEM for the baseline bars are too small to see). No significant change was observed in the *fmn* mutant flies between AHL saline (no cocaine) treatment and the three cocaine treatments (one-way ANOVA; $p = 0.9$, $n = 6$).

fly genotype exhibited a significant change in $[DA]_{max}$ from baseline dopamine measurements when only AHL saline (no cocaine) was applied in a control experiment (one-way ANOVA; $p > 0.05$, $n = 6$). Only the baseline measurements from the AHL saline control experiments (no cocaine) are plotted for simplicity. There was no significant difference between baseline measurements for wild-type and *fmn* flies that were later treated with cocaine vs AHL saline measurements for wild-type and *fmn* flies ($p > 0.05$, $n = 6$). The *fmn* mutant flies lacking the dopamine transporter exhibited no change in extracellular dopamine concentration after 0.05, 0.5, or 1.0 mM cocaine treatment (one-way ANOVA; $p = 0.9$, $n = 6$). Therefore, at the 1.0 mM concentration, cocaine appears to overcome a threshold concentration and significantly blocks the *Drosophila* dopamine transporter *in vivo*. These data are consistent with the effect of cocaine on mammalian dopamine transporter function (*5*, *8*) and with observations previously made with this technique (*38*). These findings support the use of *Drosophila* as a model system for studying pharmacology effects *in vivo*. Although the effect of volatilized cocaine on *Drosophila* behavior has previously been demonstrated (*20*), the findings presented here provide the first *in vivo* investigation of the effective cocaine concentration needed to block uptake of exogenously applied dopamine by the dopamine transporter in the adult fly.

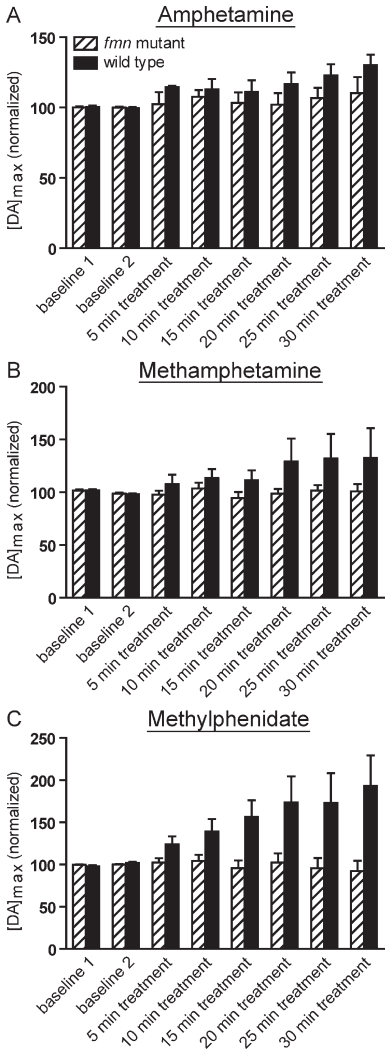

Figure 5. Comparison of uptake in adult *Drosophila* wild-type (solid) vs *fmn* mutant (striped) flies when 1.0 mM dopamine was exogenously applied before (baseline 1, 2) and after 1.0 mM stimulant treatment: (A) Following amphetamine treatment, the increases in normalized $[DA]_{max}$ are significantly higher in wild-type flies compared with *fmn* mutant flies (mean ± SEM; two-way ANOVA; $p = 0.005$ for genotype, $n = 5$). Additionally, the 30 min treatment is significantly different from baseline 2 for the wild-type flies (one-way ANOVA; $p = 0.03$, *post hoc* Tukey pairwise comparisons; $p < 0.05$). (B) The increases in normalized $[DA]_{max}$ are significantly higher in wild-type vs *fmn* flies following methamphetamine treatment (mean ± SEM; two-way ANOVA; $p = 0.01$ for genotype, $n = 5-6$). (C) Following methylphenidate treatment, the increases in normalized $[DA]_{max}$ for wild-type compared with *fmn* flies are significantly higher (mean ± SEM; two-way ANOVA; $p = 0.03$ for interaction; $p < 0.0001$ for genotype, $n = 5$).

Dopamine Uptake in Wild-Type vs *fmn* Mutant Flies Following Treatment with Stimulants

In addition to cocaine, the effects of three other stimulants on *Drosophila* dopamine transporter function were investigated. Flies were prepared as for cocaine experiments and treated with 1.0 mM amphetamine, methamphetamine, or methylphenidate in AHL saline. Figure 5 contains a summary of the normalized

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Table 1. Change in $[DA]_{max}$ for Four Drugs of Abuse[a]

	cocaine	amphetamine	methamphetamine	methylphenidate
IC_{50} (μM) *Drosophila* dopamine transporter	6.0,[b] 2.7[c]	4.9;[b] (+) 6.6, (−) 34.0[c]	4.5[b]	6.8[b]
wild type $[DA]_{max}$ (%) normalized (20 min treatment)	223 ± 40	117 ± 8	129 ± 22	174 ± 31
fmn mutant $[DA]_{max}$ (%) normalized (20 min treatment)	91 ± 8	102 ± 8	99 ± 4	102 ± 11

[a] Maximum changes for dopamine ($[DA]_{max}$) values are for (+)-amphetamine and (+)-methamphetamine. $[DA]_{max}$ values are mean ± SEM for 1.0 mM drug concentrations (n = 5−6). Literature IC_{50} values are included for comparison (*49*, *57*). [b] Reference *57*. [c] Reference *49*.

$[DA]_{max}$ for adult wild-type compared with *fmn* mutant flies following each of these drug treatments. When dopamine levels in the flies treated with amphetamine were examined over time, there was a small but significant difference in the amount of dopamine detected in the PAM region of the wild-type brain compared with the same region in the *fmn* mutant (Figure 5A; two-way ANOVA; p = 0.005 for genotype, n = 5). However, even after 30 min of treatment, the observed $[DA]_{max}$ for the amphetamine-treated wild-type flies was lower than that of wild-type flies treated with 1.0 mM cocaine (∼25% increase vs ∼125% increase). This finding is consistent with *in vitro* inhibition studies demonstrating that amphetamine is a less potent inhibitor of the *Drosophila* dopamine transporter than cocaine (*49*).

The *Drosophila* dopamine transporter was significantly affected by treatment with methamphetamine as well (Figure 5B; two-way ANOVA; p = 0.01 for genotype, n = 5−6). Methamphetamine-treated wild-type flies exhibited a similar increase in $[DA]_{max}$ compared with the amphetamine-treated wild-type flies (∼30% increase vs ∼25% increase). Interestingly, the trend in time until maximum blocking of dopamine uptake occurs is later with methamphetamine treatment than with amphetamine or cocaine treatment. Although the difference between the normalized $[DA]_{max}$ after 5 and 20 min of methamphetamine treatment in wild-type flies is not significantly different (Student's t-test, p = 0.4, n = 6), the kinetics of the action of methamphetamine on the fly dopamine transporter could be of interest in future investigations. There is *in vitro* evidence that methamphetamine and amphetamine cause internalization of the mammalian dopamine transporter. These data suggest an additional mechanism that contributes to the decrease in transporter activity by amphetamines, in addition to blocking and inducing reverse transport of dopamine through the dopamine transporter (*50*−*52*). While *in vitro* model systems are often used to predict the effects of psychostimulants on monoamine uptake, *in vitro* results are not always an accurate reflection of the potential of a compound to modulate *in vivo* function (*53*−*55*). Thus, development of analytical methods capable of *in vivo* evaluation of drug efficacy plays a critical role in the neuroscience field. Our *in vivo* measurements confirm that amphetamines do indeed alter *Drosophila* dopamine transporter function; however, with the current experimental setup, it is not possible to speculate on the exact mechanisms of action occurring in the fly CNS.

Although methylphenidate is commonly studied in mammalian systems, very little, if any, literature is available on the efficacy of this drug in *Drosophila*. Because the fruit fly is becoming a more widely used model system for studying the neurochemical basis for human behaviors and addictions (*18*), we chose to examine the effect of this commonly prescribed drug on dopamine uptake using our *in vivo* method. Following methylphenidate treatment, wild-type flies displayed a significantly higher extracellular dopamine concentration compared with baseline dopamine measurements and the treated *fmn* mutant flies (Figure 5C; two-way ANOVA; p = 0.03 for interaction, p < 0.0001 for genotype, n = 5). This indicates that methylphenidate blocks dopamine uptake occurring via the *Drosophila* dopamine transporter. This finding correlates with the proposed mechanism of methylphenidate in the human brain (*13*, *14*, *56*) and supports the use of *Drosophila* in future studies on methylphenidate. Of the four stimulants investigated, cocaine and methylphenidate displayed the greatest effect on *Drosophila* dopamine transporter function *in vivo* (Table 1).

In our experiments, exogenously applied dopamine is cleared primarily through diffusion, metabolism, and uptake by the dopamine transporter. By comparing two fly genotypes whose diffusion and metabolism are presumably similar since they only differ in dopamine transporter function, we were able to investigate the uptake component of dopamine clearance in the presence of various stimulants. All stimulants tested caused significantly increased dopamine signal amplitudes ($[DA]_{max}$), which has also been observed in the cocaine-treated rat CNS where chronoamperometry was employed to measure exogenously applied dopamine concentrations *in vivo* (*17*, *44*). In these studies, Gerhardt and co-workers reported an increase in the time course of the enhanced dopamine signal amplitudes, which we did not observe in *Drosophila*. We speculate that diffusion plays a prominent role in the clearance of dopamine from the *Drosophila* CNS due to its reduced size (∼ 5 nL) while the rat CNS might have decreased diffusion of dopamine away from the electrode (*32*). A change in $t_{1/2}$ could be too

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minor to observe in the fly system relative to this diffusion factor.

Conclusion

This study presents *in vivo* measurements of dopamine uptake using exogenously applied dopamine as a function of cocaine concentration in *Drosophila*. In addition, physiological effects of amphetamine, methamphetamine, and methylphenidate are also reported for the adult fly. Furthermore, cocaine and methylphenidate are more potent at inhibiting dopamine uptake *in vivo* by the *Drosophila* dopamine transporter than amphetamine and methamphetamine. It is most likely that the variation in our dose−response results among the four stimulants tested here reflects different interactions of the drugs with the dopamine transporter. Little is known about the *in vivo* nature of drug interactions with invertebrate transporters, mainly because of the lack of tools heretofore available for quantifying neurotransmitters in such small native environments. These data support continued use of this *in vivo Drosophila* model system to further investigate dopamine neurotransmission and enhance our understanding of the physiological mechanisms that underlie human behaviors and addictions.

Methods

Chemicals

All chemicals were used as received and purchased from Sigma (St. Louis, MO) unless otherwise stated. Adult-hemolymph-like (AHL) saline (108 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 8.2 mM $MgCl_2$, 4 mM $NaHCO_3$, 1 mM NaH_2PO_4, 5 mM trehalose (Fluka BioChemika, Buchs, Switzerland), 10 mM sucrose, 20 mM Tris, pH 7.5) was made using ultrapure (18 MΩ·cm) water and filtered through a 0.2 μm filter (*19*). All collagenase, KCl, dopamine, *N*-acetyl-*p*-aminophenol (APAP, acetaminophen), cocaine, (+)-amphetamine, (+)-methamphetamine, and methylphenidate solutions were prepared using AHL saline.

In Vivo Drosophila Preparation

The Canton-S strain of *Drosophila melanogaster* was used as the wild-type strain in this study. The *fumin* (*fmn*) mutant has a genetic lesion abolishing the dopamine transporter function. The genetic background of the w;*fmn* mutant was replaced with the Canton-S background. All flies were maintained at 25 °C on a standard cornmeal-agar medium, and 4−10-day-old male flies were used in all experiments. The flies were prepared for *in vivo* voltammetry as previously described (*38*). Briefly, flies were immobilized on ice and mounted in a homemade collar (38.1 mm diameter concave plexiglass disk with 1.0 mm hole in center) with low melting agarose (Fisher Scientific, Pittsburgh, PA). Microsurgery was performed on a stereoscope (Olympus SZ60, Melville, NY). After the cuticle was removed from the top portion of the head to expose the brain, the head was covered with 0.1% collagenase solution for 30 min to relax the extracellular matrix in the brain. The head of the immobilized fly was then rinsed and bathed with AHL saline ("bath application method") with the preparation maintaining its viability for 1.5−2.5 h.

Electrochemical Measurements

Carbon-fiber microelectrodes were fabricated as previously described (*58*). Briefly, a single 5 μm diameter carbon fiber (Amoco, Greenville, SC) was aspirated into a borosilicate glass capillary (B120-69-10, Sutter Instruments, Novato, CA), and the capillary was pulled using a regular glass capillary puller (P-97, Sutter Instruments). Electrical contact was made by backfilling the capillary with silver composition (4922N DuPont, Delta Technologies Ltd., Stillwater, MN) and inserting a tungsten wire. To form a cylindrical electrode, the carbon fiber was cut to a length of 40−50 μm, as measured from the glass junction. Electrode tips were dipped into epoxy (Epo-Tek, Epoxy Technology, Billerica, MA) for 30 s to ensure a good seal between the fiber and the glass and then dipped into acetone for 15 s to remove epoxy from exposed carbon fiber. Standard dopamine solutions were used for *in vitro* electrode calibration as previously described (*38*). A silver wire (0.25 mm diameter, 99.999% purity, Alfa Aesar, Ward Hill, MA) was chloridized in bleach overnight, and the Ag/AgCl electrode served as the reference electrode in all experiments. All electrodes were positioned using micromanipulators (421 series, Newport, Irvine, CA). Micropipet injectors were fabricated by pulling glass capillaries in a glass capillary puller to an opening of approximately 5 μm. Micropipet injectors were coupled to the microinjection system (Picospritzer II, General Valve Corporation, Fairfield, NJ) and used to exogenously apply dopamine solution.

Electrochemical data were collected using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) or a Dagan Chem-Clamp potentiostat (Dagan Corporation, Minneapolis, MN) and two data acquisition boards (PCI-6221, National Instruments, Austin, TX) run by the TH 1.0 CV program (ESA, Chelmsford, MA) (*36*). All cyclic voltammograms were obtained using a triangular waveform (scanned −0.6 V to +1.0 V versus Ag/AgCl at 200 V/s) repeated every 100 ms. Prior to voltammetric experiments, all electrodes were cycled (−0.6 V to +1.0 at 200 V/s) for at least 15 min to stabilize the background current. Electrochemical responses were plotted and statistical analysis performed using Prism 5.0 (GraphPad Software, La Jolla, CA).

Supporting Information Available

APAP bath application. This information is available free of charge via the Internet at http://pubs.acs.org/.

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References

1. Chen, N., and Reith, M. E. A. (2000) Structure and function of the dopamine transporter. *Eur. J. Pharmacol. 405*, 329–339.

2. Mortensen, O. V., and Amara, S. G. (2003) Dynamic regulation of the dopamine transporter. *Eur. J. Pharmacol. 479*, 159–170.

3. Di Chiara, G., and Imperato, A. (1988) Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proc. Natl. Acad. Sci. U.S.A. 85*, 5274–5278.

4. Kuhar, M. J., Ritz, M. C., and Boja, J. W. (1991) The dopamine hypothesis of the reinforcing properties of cocaine. *Trends Neurosci. 14*, 299–302.

5. Giros, B., Jaber, M., Jones, S. R., Wightman, R. M., and Caron, M. G. (1996) Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. *Nature 379*, 606–612.

6. Ritz, M. C., Lamb, R. J., Goldberg, S. R., and Kuhar, M. J. (1987) Cocaine receptors on dopamine transporters are related to self-administration of cocaine. *Science 237*, 1219–1223.

7. Carboni, E., Spielewoy, C., Vacca, C., Nosten-Bertrand, M., Giros, B., and Di Chiara, G. (2001) Cocaine and amphetamine increase extracellular dopamine in the nucleus accumbens of mice lacking the dopamine transporter gene. *J. Neurosci. 21*, 141RC.

8. Sora, I., Hall, F. S., Andrews, A. M., Itokawa, M., Li, X.-F., Wei, H.-B., Wichems, C., Lesch, K.-P., Murphy, D. L., and Uhl, G. R. (2001) Molecular mechanisms of cocaine reward: Combined dopamine and serotonin transporter knockouts eliminate cocaine place preference. *Proc. Natl. Acad. Sci. U.S.A. 98*, 5300–5305.

9. Kahlig, K. M., Binda, F., Khoshbouei, H., Blakely, R. D., McMahon, D. G., Javitch, J. A., and Galli, A. (2005) Amphetamine induces dopamine efflux through a dopamine transporter channel. *Proc. Natl. Acad. Sci. U.S.A. 102*, 3495–3500.

10. Sulzer, D., Chen, T., Lau, Y., Kristensen, H., Rayport, S., and Ewing, A. (1995) Amphetamine redistributes dopamine from synaptic vesicles to the cytosol and promotes reverse transport. *J. Neurosci. 15*, 4102–4108.

11. Jones, S. R., Gainetdinov, R. R., Wightman, R. M., and Caron, M. G. (1998) Mechanisms of amphetamine action revealed in mice lacking the dopamine transporter. *J. Neurosci. 18*, 1979–1986.

12. Carrey, N. J., Wiggins, D. M., and Milin, R. P. (1996) Pharmacological treatment of psychiatric disorders in children and adolescents - Focus on guidelines for the primary care practitioner. *Drugs 51*, 750–759.

13. Volkow, N. D., Wang, G.-J., Fowler, J. S., Gatley, S. J., Logan, J., Ding, Y.-S., Hitzemann, R., and Pappas, N. (1998) Dopamine transporter occupancies in the human brain induced by therapeutic doses of oral methylphenidate. *Am. J. Psychiatry 155*, 1325–1331.

14. Volkow, N. D., Wang, G.-J., Fowler, J. S., Logan, J., Gerasimov, M., Maynard, L., Ding, Y.-S., Gatley, S. J., Gifford, A., and Franceschi, D. (2001) Therapeutic doses of oral methylphenidate significantly increase extracellular dopamine in the human brain. *J. Neurosci. 21*, 121RC.

15. Volkow, N. D., Ding, Y.-S., Fowler, J. S., Wang, G.-J., Logan, J., Gatley, J. S., Dewey, S., Ashby, C., Liebermann, J., Hitzemann, R., and Wolf, A. P. (1995) Is methylphenidate like cocaine? Studies on their pharmacokinetics and distribution in the human brain. *Arch. Gen. Psychiatry 52*, 456–463.

16. Balster, R. L., and Schuster, C. R. (1973) Fixed-interval schedule of cocaine reinforcement - effect of dose and infusion duration. *J. Exp. Anal. Behav. 20*, 119–129.

17. Zahniser, N. R., Larson, G. A., and Gerhardt, G. A. (1999) In vivo dopamine clearance rate in rat striatum: Regulation by Extracellular dopamine concentration and dopamine transporter inhibitors. *J. Pharmacol. Exp. Ther. 289*, 266–277.

18. Makos, M. A., Kuklinski, N. J., Berglund, E. C., Heien, M. L., Ewing, A. G. (2009) Chemical measurements in Drosophila, *Trends Anal. Chem.* in press. doi: 10.1016/j.trac.2009.08.005.

19. Wang, J. W., Wong, A. M., Flores, J., Vosshall, L. B., and Axel, R. (2003) Two-photon calcium imaging reveals an odor-evoked map of activity in the fly brain. *Cell 112*, 271–282.

20. Li, H., Chaney, S., Forte, M., and Hirsh, J. (2000) Ectopic G-protein expression in dopamine and serotonin neurons blocks cocaine sensitization in *Drosophila melanogaster*. *Curr. Biol. 10*, 211–214.

21. Schultz, W. (2000) Multiple reward signals in the brain. *Nat. Rev. Neurosci. 1*, 199–207.

22. Waddell, S., and Quinn, W. G. (2001) What can we teach *Drosophila*? What can they teach us? *Trends Genet. 17*, 719–726.

23. Kim, Y. C., Lee, H. G., and Han, K. A. (2007) Classical reward conditioning in *Drosophila melanogaster*. *Genes Brain Behav. 6*, 201–207.

24. Bainton, R. J., Tsai, L. T. Y., Singh, C. M., Moore, M. S., Neckameyer, W. S., and Heberlein, U. (2000) Dopamine modulates acute responses to cocaine, nicotine and ethanol in *Drosophila*. *Curr. Biol. 10*, 187–194.

25. McClung, C., and Hirsh, J. (1998) Stereotypic behavioral responses to free-base cocaine and the development of behavioral sensitization in *Drosophila*. *Curr. Biol. 8*, 109–112.

DOI: 10.1021/cn900017w | *ACS Chem. Neurosci.* (2010), *1*, 74–83

26. Rothenfluh, A., and Heberlein, U. (2002) Drugs, flies, and videotape: The effects of ethanol and cocaine on *Drosophila* locomotion. *Curr. Opin. Neurobiol. 12*, 639–645.

27. Torres, G., and Horowitz, J. M. (1998) Activating properties of cocaine and cocaethylene in a behavioral preparation of *Drosophila melanogaster*. *Synapse 29*, 148–161.

28. Chang, H. Y., Grygoruk, A., Brooks, E. S., Ackerson, L. C., Maidment, N. T., Bainton, R. J., and Krantz, D. E. (2006) Overexpression of the *Drosophila* vesicular monoamine transporter increases motor activity and courtship but decreases the behavioral response to cocaine. *Mol. Psychiatry 11*, 99–113.

29. Nathanson, J. A., Hunnicutt, E. J., Kantham, L., and Scavone, C. (1993) Cocaine as a naturally occurring insecticide. *Proc. Natl. Acad. Sci. U.S.A. 90*, 9645–9648.

30. Ewing, A. G., Bigelow, J. C., and Wightman, R. M. (1983) Direct in vivo monitoring of dopamine released from 2 striatal compartments in the rat. *Science 221*, 169–171.

31. Ewing, A. G., and Wightman, R. M. (1984) Monitoring the stimulated release of dopamine with in vivo voltammetry. II: Clearance of released dopamine from extracellular fluid. *J. Neurochem. 43*, 570–577.

32. Sabeti, J., Adams, C. E., Burmeister, J., Gerhardt, G. A., and Zahniser, N. R. (2002) Kinetic analysis of striatal clearance of exogenous dopamine recorded by chronoamperometry in freely-moving rats. *J. Neurosci. Methods 121*, 41–52.

33. Michael, D., Travis, E. R., and Wightman, R. M. (1998) Color images for fast-scan CV. *Anal. Chem. 70*, 586a–592a.

34. Baur, J. E., Kristensen, E. W., May, L. J., Wiedemann, D. J., and Wightman, R. M. (1988) Fast-scan voltammetry of biogenic-amines. *Anal. Chem. 60*, 1268–1272.

35. Clark, R. A., Zerby, S. E., Ewing, A. G. (1996) Electrochemistry in Neuronal Microenvironments, in *Electroanalytical Chemistry: A Series of Advances* (Bard, A. J., Rubinstein, I., Eds.), pp 227−295, Marcel Dekker, New York.

36. Heien, M. L. A. V., Phillips, P. E. M., Stuber, G. D., Seipel, A. T., and Wightman, R. M. (2003) Overoxidation of carbon-fiber microelectrodes enhances dopamine adsorption and increases sensitivity. *Analyst 128*, 1413–1419.

37. Borue, X., Cooper, S., Hirsh, J., Condron, B., and Venton, B. J. (2009) Quantitative evaluation of serotonin release and clearance in *Drosophila*. *J. Neurosci. Methods 179*, 300–308.

38. Makos, M. A., Kim, Y.-C., Han, K.-A., Heien, M. L., and Ewing, A. G. (2009) In vivo electrochemical measurements of exogenously applied dopamine in *Drosophila melanogaster*. *Anal. Chem. 81*, 1848–1854.

39. Coulom, H., and Birman, S. (2004) Chronic exposure to rotenone models sporadic Parkinson's disease in *Drosophila melanogaster*. *J. Neurosci. 24*, 10993–10998.

40. Zhang, K., Guo, J. Z., Peng, Y., Xi, W., and Guo, A. (2007) Dopamine-mushroom body circuit regulates saliency-based decision-making in *Drosophila*. *Science 316*, 1901–1904.

41. Nassel, D. R., and Elekes, K. (1992) Aminergic neurons in the brain of blowflies and *Drosophila*: Dopamine- and tyrosine hydroxylase-immunoreactive neurons and their relationship with putative histaminergic neurons. *Cell Tissue Res. 267*, 147–167.

42. Kim, Y.-C., Lee, H.-G., and Han, K.-A. (2007) D1 Dopamine receptor dDA1 is required in the mushroom body neurons for aversive and appetitive learning in Drosophila. *J. Neurosci. 27*, 7640–7647.

43. Millar, J., Stamford, J. A., Kruk, Z. L., and Wightman, R. M. (1985) Electrochemical, pharmacological and electrophysiological evidence of rapid dopamine release and removal in the rat caudate nucleus following electrical stimulation of the median forebrain bundle. *Eur. J. Pharmacol. 109*, 341–348.

44. Cass, W. A., Zahniser, N. R., Flach, K. A., and Gerhardt, G. A. (1993) Clearance of exogenous dopamine in rat dorsal striatum and nucleus accumbens: Role of metabolism and effects of locally applied uptake inhibitors. *J. Neurochem. 61*, 2269–2278.

45. Church, W. H., Justice, J. B., Jr, and Byrd, L. D. (1987) Extracellular dopamine in rat striatum following uptake inhibition by cocaine, nomifensine and benztropine. *Eur. J. Pharmacol. 139*, 345–348.

46. Morrison, P. F., Bungay, P. M., Hsiao, J. K., Ball, B. A., Mefford, I. N., and Dedrick, R. L. (1991) Quantitative microdialysis - analysis of transients and application to pharmacokinetics in brain. *J. Neurochem. 57*, 103–119.

47. Logman, M. J., Budygin, E. A., Gainetdinov, R. R., and Wightman, R. M. (2000) Quantitation of in vivo measurements with carbon fiber microelectrodes. *J. Neurosci. Methods 95*, 95–102.

48. Miner, D. J., Rice, J. R., Riggin, R. M., and Kissinger, P. T. (1981) Voltammetry of acetaminophen and its metabolites. *Anal. Chem. 53*, 2258–2263.

49. Pörzgen, P., Park, S. K., Hirsh, J., Sonders, M. S., and Amara, S. G. (2001) The antidepressant-sensitive dopamine transporter in *Drosophila melanogaster*: A primordial carrier for catecholamines. *Mol. Pharmacol. 59*, 83–95.

50. Saunders, C., Ferrer, J. V., Shi, L., Chen, J., Merrill, G., Lamb, M. E., Leeb-Lundberg, L. M. F., Carvelli, L., Javitch, J. A., and Galli, A. (2000) Amphetamine-induced loss of human dopamine transporter activity: An internalization-dependent and cocaine-sensitive mechanism. *Proc. Natl. Acad. Sci. U.S.A. 97*, 6850–6855.

51. Melikian, H. E., and Buckley, K. M. (1999) Membrane trafficking regulates the activity of the human dopamine transporter. *J. Neurosci. 19*, 7699–7710.

52. Sandoval, V., Riddle, E. L., Ugarte, Y. V., Hanson, G. R., and Fleckenstein, A. E. (2001) Methamphetamine-induced rapid and reversible changes in dopamine transporter function: an in vitro model. *J. Neurosci. 21*, 1413–1419.

53. Fleckenstein, A. E., Haughey, H. M., Metzger, R. R., Kokoshka, J. M., Riddle, E. L., Hanson, J. E., Gibb, J. W., and Hanson, G. R. (1999) Differential effects of psychostimulants and related agents on dopaminergic and serotonergic transporter function. *Eur. J. Pharmacol. 382*, 45–49.

DOI: 10.1021/cn900017w | *ACS Chem. Neurosci.* (2010), *1*, 74–83

54. Kilty, J., Lorang, D., and Amara, S. (1991) Cloning and expression of a cocaine-sensitive rat dopamine transporter. *Science 254*, 578–579.

55. Richelson, E., and Pfenning, M. (1984) Blockade by antidepressants and related compounds of biogenic amine uptake into rat brain synaptosomes: Most antidepressants selectively block norepinephrine uptake. *Eur. J. Pharmacol. 104*, 277–286.

56. Sora, I., Wichems, C., Takahashi, N., Li, X.-F., Zeng, Z., Revay, R., Lesch, K.-P., Murphy, D. L., and Uhl, G. R. (1998) Cocaine reward models: Conditioned place preference can be established in dopamine- and in serotonin-transporter knockout mice. *Proc. Natl. Acad. Sci. U.S.A. 95*, 7699–7704.

57. Chen, R., Wei, H., Hill, E., Chen, L., Jiang, L., Han, D., and Gu, H. (2007) Direct evidence that two cysteines in the dopamine transporter form a disulfide bond. *Mol. Cell. Biochem. 298*, 41–48.

58. Dayton, M. A., Brown, J. C., Stutts, K. J., and Wightman, R. M. (1980) Faradaic electrochemistry at micro-voltammetric electrodes. *Anal. Chem. 52*, 946–950.

DOI: 10.1021/cn900017w | *ACS Chem. Neurosci.* (2010), *1*, 74–83