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The membrane-raft protein Flotillin-1 is essential in dopamine neurons for amphetamine-induced behavior in *Drosophila*

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Abstract

The dopamine transporter (DAT) is the primary molecular target responsible for the rewarding properties of the psychostimulants amphetamine (AMPH) and cocaine. AMPH increases

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Author Contribution

ABP, CSK and JAJ designed the experiments, analyzed the data, discussed the results, and wrote the manuscript. ABP generated the hDAT mutant transgenic flies, developed the experimental protocol and implemented hardware setups for the behavioral tests. ABP, CSK and YZ performed the experiments. HY generated the hFlot1 and hFlot1(C34A) transgenic flies. RF performed the statistical analyses. DSK developed the code that automates Choreography. BDM provided advice on fly genetics and the development of the behavioral assay. ZF, AY and BDM discussed the results and commented on the manuscript.

Competing Financial Interests

The authors declare no competing financial interests.

extracellular dopamine (DA) by promoting its nonexocytotic release via DAT-mediated efflux. Previous studies in heterologous cells have shown that phosphorylation of the amino terminus of DAT is required for AMPH-induced DA efflux but not for DA uptake. However, the identity of many of the modulatory proteins and the molecular mechanisms that coordinate efflux and the ensuing behavioral effects remain poorly defined. Here we establish a robust assay for AMPH-induced hyperlocomotion in *Drosophila melanogaster* larvae. Using a variety of genetic and pharmacological approaches we demonstrate that this behavioral response is dependent on DA and on DAT and its phosphorylation. We also show that methylphenidate (MPH), which competitively inhibits DA uptake but does not induce DAT-mediated DA efflux, also leads to DAT-dependent hyperlocomotion, but this response is independent of DAT phosphorylation. Moreover, we demonstrate that the membrane raft protein Flotillin1 is required for AMPH-induced but not MPH-induced hyperlocomotion. These results are the first evidence of a role for a raft protein in an AMPH-mediated behavior. Thus, using our assay we are able to translate molecular and cellular findings to a behavioral level and to differentiate *in vivo* the distinct mechanisms of two psychostimulants.

Abuse of psychostimulants is a major public health problem with profound psychiatric, medical and psychosocial complications, for which no effective treatments currently exist.[1] Psychostimulants such as amphetamines (AMPHs), cocaine, and methylphenidate (MPH; Ritalin®) target multiple biogenic amine transporters, resulting in elevated extracellular levels of the cognate neurotransmitters. AMPH and methamphetamine (METH) are substrates for the dopamine (DA), norepinephrine, and serotonin transporters (DAT, NET, and SERT, respectively).[2] Once accumulated inside cells, these compounds lead to depletion of the endogenous neurotransmitters from synaptic vesicles via action at the vesicular monoamine transporter 2 (VMAT2), and induce reverse transport (efflux) of the neurotransmitters mediated by the plasma membrane transporters, culminating in massive non-exocytotic release.[3] In contrast, cocaine and MPH lead to elevated extracellular levels of the biogenic amines through competitive inhibition of their reuptake by DAT, NET and SERT, without stimulating efflux.[4]

Multiple lines of evidence suggest that DAT is the primary molecular target for the rewarding properties and abuse liability of psychostimulants[3, 5-12], although studies in genetically modified mice have suggested a potential role for SERT and NET as well.[11,13-16] DAT knockout mice, which exhibit high extracellular DA levels and marked hyperactivity, show a “paradoxical” inhibitory locomotor response to AMPH, as they do to cocaine[13-15], and it has been proposed that this is due to compensatory developmental changes in serotonin signaling in the setting of dramatically altered DA signaling.[14] Perhaps the most clear-cut data without such a developmental confound come from a knock-in mouse expressing a functional DAT mutant that transports DA at wild-type levels but has a greatly reduced affinity for cocaine[17] and MPH.[18] This mouse shows significantly reduced hyperlocomotion in response to cocaine and MPH, as well as impaired cocaine self-administration[19] and conditioned place preference[17, 18], measures of drug-induced reward. These studies indicate that DAT is indeed the essential target for the rewarding properties of cocaine and MPH. Still, there is room for complex polygenetic determinants of psychostimulant function, involving other transporters, receptors and regulatory proteins.

Furthermore, as no analogous AMPH-resistant transgenic mice exist, it has been difficult to delineate the role of the various biogenic amine transporters and other regulatory proteins in the behavioral effects of AMPH *in vivo*.

A number of interacting proteins and signaling pathways are known to mediate the effects of AMPH on neurotransmitter transporter function.[5] Mutation or deletion of serine residues in the distal N-terminus of DAT severely reduces AMPH-induced DA efflux *in vitro*, whereas neither manipulation has a significant effect on the uptake function of DAT.[20, 21] These data suggest that phosphorylation of DAT plays an essential role in its ability to efflux DA in response to AMPH without affecting its transport capacity. Furthermore, in cellular studies we recently identified the membrane raft-associated protein Flotillin-1 (Flot1) as a novel regulator of DAT function.[22] Flot1 is required to maintain the localization of DAT in cholesterol-rich regions in the plasma membrane. Depletion of Flot1 severely blunts AMPH-induced DA efflux without impairing DAT-mediated DA uptake in cells in culture.[22] Taken together, these studies show that the mechanisms modulating AMPH-mediated DA efflux are distinct from those that govern DA transport. Still, the extent to which these mechanisms contribute to the behavioral properties of AMPH remains to be determined.

With its accessibility to genetic and molecular analyses, *Drosophila melanogaster* provides a powerful model system to address these phenomena more broadly and efficiently.[23, 24] Key mechanisms of synaptic neurotransmission are conserved between flies and humans. Biogenic amine neurotransmitters such as DA and serotonin are present in *Drosophila*.[25-27] They regulate similar functions to those in mammals, including circadian rhythms[28], aggression[29, 30], attention[31], reward, learning and memory.[32, 33] DA has also been shown to modulate changes in locomotion induced by drugs such as METH[34], cocaine[35-37], caffeine[38] and ethanol.[36, 39] While the adrenergic neurotransmitters norepinephrine and epinephrine have not been identified in *Drosophila*, the structurally-related phenolamines octopamine (OA) and tyramine (TA)[26, 40] have been shown to play analogous roles in locomotion[41] and the response to cocaine.[42] The fly homologues of most, if not all, molecules involved in monoaminergic synaptic transmission, such as synthesis enzymes, receptors, and transporters have been identified.[43, 44] These studies suggest that *Drosophila* can provide a powerful and tractable genetic model to study the molecular determinants of psychostimulant-induced behavior.

Here, we establish a novel locomotor assay in *Drosophila* larvae to investigate mechanisms of DAT-mediated psychostimulant action. We show that larvae display DAT-dependent hyperlocomotion in response to acute administration of either AMPH or MPH. Phosphorylation of the DAT N-terminus is required for AMPH-induced but not MPH-induced hyperlocomotion. Furthermore, we show that Flot1 is required in DA neurons for the AMPH-induced response, suggesting a critical role for membrane rafts as integrators of signaling that is essential to AMPH-induced behavior. In contrast, Flot1 is not required for the response to MPH. Our findings thus establish a robust experimental system that reveals novel insights into the molecular processes underlying the behavioral response to two psychostimulants with distinct mechanisms of action on DA pathways *in vivo*.

Materials and Methods

Fly Stocks and Transgenic *Drosophila* Lines

All fly strains were raised at 25 °C and 60% humidity on standard cornmeal-molasses medium on a 12:12 h light/dark schedule. An isogenic w^{1118} fly strain (Exelixis strain A5001, BL-6326) was used as the wild-type control.

Transgenic strains *UAS-hDAT, UAS-hFlot1* and *UAS-hFlot1C34A* were generated in the same w^{1118} background. pBI-UAS expression vectors[45] were used to clone Venus-hDAT, hFlot1-mRFP and hFlot1C34A-mRFP and phiC31 targeted germ line transformation was performed at the Model System Genomics Facility at Duke University. The Venus-hDAT, Venus-hDAT-S/A and Venus-hDAT-S/D transgenes were inserted into the ZH-attP-96E site on chromosome 3. The hFlot1-mRFP and hFlot1C34A-mRFP transgenes were both inserted into the ZH-attp-2A site on the X chromosome. Expression of the fluorescent fusion proteins in DA neurons using the TH-GAL4 driver was confirmed by confocal microscopy (data not shown). Comparative analyses have previously shown that dDAT and hDAT share 49% overall sequence identity, with ~80% similarity in the transmembrane domains.[43] Flo and hFlot1 share ~60% sequence identity, with ~80% similarity at the amino acid level.[46] The sequence of the RNAi against Flo used in the study does not target hFlot1.

The $dDAT^{fmn}$ mutants were a gift from Dr. K. Kume (Kumamoto, Japan) and were back-crossed to the w^{1118} wild-type strain for 7 generations. These mutants have the 5′ portion of a roo transposon inserted into intron 6 of the *dDAT* gene resulting in an in-frame stop codon.[28] The Flo^{e02554} mutants were obtained from the Exelixis Collection at Harvard University (Boston, MA, USA) and were back-crossed to the w^{1118} wild-type strain for 7 generations. These mutants have a piggyBac transposon inserted into intron 2 of the *Flo* gene[47] as confirmed by PCR (Supplementary Fig. 1). $Tdc2^{RO54}$ flies were a gift from Dr. Mariana Wolfner (Ithaca, NY, USA). These mutants carry an EMS-induced point mutation which results in a glutamic acid to lysine substitution in the pyridoxal-dependent decarboxylase domain.[42, 48] The *DTHg; ple* and $DTHg^{FS\pm}$*; ple*, strains were a gift from Dr. S. Birman (Paris, France).[49] *DTHg; ple* allowed for the full rescue of the null dTH mutation *(ple)* by expressing genomic dTH using both TH-GAL4 and Ddc-GAL4 (also a gift from Dr. Birman). The $DTHg^{FS\pm}$*; ple* allowed for rescue of the ple mutation only in non-neuronal cells, using the same GAL4 driver lines. DAT RNAi (line 106961) and Flo RNAi (line 42130) transgenic strains were obtained from the Vienna *Drosophila* RNAi Center (VDRC, Vienna, Austria). GFP RNAi (line 9330) and TH RNAi (line 25796) transgenic strain were obtained from Bloomington Stock Center. All transgene lines were driven by the TH-GAL4 driver (unless otherwise indicated), a gift from Dr. S. Birman (Paris, France). Dicer2 (VDRC line 60008) was over-expressed with all RNAi transgenes to enhance the efficiency of RNAi knockdown.[50]

Behavioral Assay

Two hundred flies (3:2, females:males) were allowed to lay eggs for 3 days in bottles filled with standard medium. Experiments were performed on the fourth day after the beginning of egg-laying. On the day of the experiment, 20-30 early third instar feeding larvae (82-86

hours old) per group were washed with distilled water, selected by size using a brush, and placed on top of 70% yeast paste or 70% yeast paste in 60 mM AMPH (Sigma, A5880) or 3 mM MPH (Sigma, M2892 or NIDA drug supply program) (unless otherwise indicated) for 1 h (unless otherwise indicated) at 18 °C. Mianserin (Sigma, M2525) (0.8 mM) was used as vehicle, unless otherwise indicated. Food dye was added to the yeast paste in order to determine whether larvae fed.

Data Acquisition and Analysis

The assay was performed on 100 mm Petri dishes filled with 1% agar dissolved in distilled water. For each set of 1-3 animals, a fresh agar plate was positioned on a cool-operating, evenly illuminated fluorescent light box situated beneath a video camera (Dalsa PT-41-04M60), equipped with a macro lens, and connected to a computer. The lens aperture was adjusted to capture a high contrast video image of a dark larval profile against a featureless bright background. After being allowed to feed for 1 h (unless otherwise indicated), early third instar larvae were removed from the yeast paste, washed briefly with distilled water, and then transferred carefully with a brush to the center of an agar plate. Larvae were allowed to acclimate to the agar plate for 1 min prior to data acquisition. Data were acquired for a period of 1 min (unless otherwise indicated). All assays were performed in a designated behavior room under normal ambient light, at room temperature (23-25 °C) and at 35-40% humidity.

Larvae were tracked utilizing the Multi-Worm Tracker (MWT)[51], a set of software that is capable of simultaneously tracking the behavior of multiple small, moving, high-contrast objects against a static background in real-time. It provides summary data for a population in real-time and saves a set of simple parameters for each larva at each time point that can be used for additional analysis.

Output files were analyzed using Choreography, a Java program included with the MWT that produces refined summary data from the saved parameter files generated during real-time tracking. The average speed of all larvae on one plate was calculated every second for 60 seconds and the average of these values was taken as the overall crawling speed per plate. Choreography analysis was automated using a Java data processing program, in conjunction with the Apache Commons Math Library, for high-throughput statistical functionality. Subsequent data analysis was performed using SPSS (see below).

Data obtained using MWT were initially validated by comparing them to data obtained with the Dynamic Image Analysis System (DIAS), which has been more commonly used to track larval locomotion.[41] The same assay was performed on MWT and DIAS systems and they both yielded very similar results (Supplementary Table 1). We used MWT to analyze all reported assays since several larvae could be assayed simultaneously on a single plate, thereby allowing more rapid and higher-throughput data collection and analysis.

Statistical Analysis of Behavioral Data

Boxplots represent the median as the middle line, with the lower and upper edges of the boxes representing the 25% and 75% quartiles, respectively, and the whiskers representing the 5% and 95% quantiles. In Fig. 2c, data points represent mean velocity +/− SEM.

Alteration in locomotion in response to drug treatment was reported as percent change in mean velocity. Asterisks indicate the statistical significance of the difference in velocities of larvae fed drug as compared to larvae of the same genotype fed vehicle as determined using the non-parametric Mann-Whitney-Wilcoxon (MWW) test and an alpha (*P*) value of 0.05 using SPSS (version 18.0).

PCR Analysis to Verify Flo^{e02554} Insertion Mutation

To validate the insertion of a piggyBac RB transposable element in the *Flo* gene[47], whole fly genomic DNA was isolated from wild-type (w^{1118}) or homozygous Flo^{e02554} adult flies using Puregene Core Kit B (Qiagen), according to the manufacturer's instructions. Primers were designed to detect the presence of the piggyBac RB transposon insertion in the second intron of the *Flo* gene. PCR products were amplified from genomic DNA (150-200 ng) using Hot-Start Taq™ DNA Polymerase (Denville Scientific) and the following primers: *Flo*S1: 5′-AGGACGAGCATTCGTTTGGCC-3′ *Flo*S2: 5′-AACAAGGCTACCTGTGGATCGGATAA-3′ *Flo*AS: 5′-AATCAGAGGCAGAGATCAACCACATC-3′ *piggyBac*AS: 5′-CATCTCAGTCGCCGCTTGGA-3′ *rp49*S: 5′-TACAGGCCCAAGATCGTGAA-3′ *rp49*AS: 5′-TCCAAGAAGCGCAAGGAGA-3′ PCR conditions were: 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing primers for 1 min and extension phase at 72 °C for 1 min, followed by an elongations step of 72 °C for 10 min on the last cycle. Annealing temperatures were 59 °C for FloS1/FloAS, 58 °C for FloS1/ piggyBacAS, 59 °C for FloS1/piggyBacAS, and 53 °C for rp49S/ rp49AS.

Results

A behavioral assay for amphetamine-induced hyperlocomotion in *Drosophila* larvae

Behavioral studies in rodents[11] and *C. elegans*[52] have shown that phenotypic changes in locomotion are reliable indicators of AMPH-induced signaling. To establish a model for AMPH action in *Drosophila*, we quantified the effect of acute AMPH treatment on the locomotion of early third instar larvae (82-86 hours old) (for a detailed description of the assay see Materials and Methods). Larvae were fed yeast suspended in either water or an AMPH solution. After 1 h, larvae were removed from the yeast paste and placed in sets of 2-3 animals into a fresh agar-coated plate positioned on a light box situated beneath a video camera. The animals were allowed to acclimate to the new environment for 1 min prior to data acquisition. Locomotor activity was then tracked using the Multi-Worm Tracker (MWT), a real-time computer vision system developed for *C. elegans*[51] and adapted for *Drosophila* larvae[53], which allows simultaneous behavioral tracking of multiple animals. The average speed of locomotion over 1 min was determined for each set of larvae. Using this assay, we observed that wild-type larvae (w^{1118}) fed yeast suspended in AMPH solution for 1 h displayed a dose-dependent increase in crawling speed. This hyperlocomotion was significant at a dosage of 20 mM AMPH (24%, $P < 0.001$) and persisted at 60 mM (21%, $P < 0.001$) and 80 mM (17%, $P < 0.01$) (Fig. 1a). These data established that, like rodents and worms, *Drosophila* larvae display a locomotor response to acute AMPH treatment.

Tyramine and octopamine mediate basal larval locomotion but are not required for amphetamine-induced hyperlocomotion

The biogenic amines tyramine (TA) and octopamine (OA) have been shown to play major roles in *Drosophila* larval locomotion; TA inhibits whereas OA stimulates basal locomotion.[41, 42] The neuronal tyrosine decarboxylase 2 (Tdc2) catalyzes the first step in the biosynthesis of TA, with TA acting as the precursor of OA[54] (Supplementary Fig. 2). *Drosophila* larvae lacking neural TA and OA due to a loss-of-function mutation in *Tdc2* (*Tdc2*^{RO54})[48, 54] have dramatically reduced basal locomotor activity levels and are hypersensitive to cocaine.[42] To examine the potential involvement of TA/OA neurotransmission in AMPH-induced hyperlocomotion, we fed *Tdc2*^{RO54} mutant larvae 60 mM AMPH and assayed them as described above. Consistent with previous findings[42], the basal locomotor speed of *Tdc2*^{RO54} mutants was dramatically reduced as compared to wild-type (*w*^{1118}) larvae. In contrast, AMPH-induced hyperlocomotion was not impaired, as *Tdc2*^{RO54} mutant larvae displayed a robust increase in velocity (65%, $P < 0.001$) when fed AMPH (Fig. 1b, Supplementary Table 2). These data establish that TA and OA are not required for AMPH-induced hyperlocomotion.

Dopamine does not influence basal larval locomotion but is essential for amphetamine-induced hyperlocomotion

To determine whether DA is required for the response to AMPH in *Drosophila*, we analyzed locomotor activity in larvae with diminished levels of tyrosine hydroxylase (TH). TH catalyzes the first and rate-limiting step in the biosynthesis of DA through the oxidation of L-tyrosine to 3,4-dihydroxy-L-phenylalanine (L-Dopa) (Supplementary Fig. 2). The *Drosophila TH* gene (*dTH*) is expressed in neural and hypodermal DA-synthesizing cells. Null mutations in the gene are embryonic lethal, as DA is required as a precursor substrate during cuticle development.[49, 55] We used targeted RNA-mediated interference (RNAi) to deplete TH using the TH-GAL4 driver[56] (*TH-GAL4*/*UAS-TH-RNAi*). The TH-GAL4 transgene, which has been extensively used as a dopaminergic driver in behavioral studies, is expressed in most, but not all, DA neurons.[56-58] Nevertheless, we refer to the TH-GAL4 positive cells as DA neurons for simplicity. Larvae expressing TH-RNAi driven by TH-GAL4 exhibit pigmentation defects and fail to pupate[59]; however, some survive to the third instar, allowing us to test them in our assay. We found that decreasing levels of TH expression did not affect basal locomotion, as the basal crawling speed of these larvae was not significantly different from control (*w*^{1118}) larvae. This is consistent with previous studies showing that profound inhibition of DA synthesis resulting from feeding 3-iodotyrosine did not impair basal locomotion of wild-type larvae.[60] In contrast, knockdown of TH blunted AMPH-induced hyperlocomotion, as TH-deficient larvae did not change their speed of locomotion when fed 60 mM AMPH (Fig. 1b, Supplementary Table 2). These data demonstrated that while DA does not play a significant role in basal larval locomotion, it is essential for AMPH-induced hyperlocomotion.

Pharmacological inhibition of octopamine neurotransmission enhances the fractional increase in speed in response to amphetamine

Our analysis of *Tdc2* mutants suggested that inhibiting OA signaling could enhance the signal to noise ratio in our measurements of the larval locomotor response to AMPH and thereby enhance the robustness of our assay. Mianserin has been used as an OA receptor antagonist in various insect models.[26, 61-64] In *Drosophila*, 0.6 mM mianserin in food has been shown to inhibit the effect of octopamine on nighttime sleep.[63] We hypothesized that treatment with mianserin, like mutation of *Tdc2*, would lead to a decrease in the speed of locomotion and to a larger fractional increase in speed in response to AMPH. Indeed, wild-type larvae (w^{1118}) fed yeast suspended in mianserin for 1 h exhibited a dose-dependent reduction in crawling speed (Supplementary Fig. 3). Larvae with depleted TH in DA neurons (*TH-GAL4*/*UAS-TH-RNAi*) exhibited a reduction in crawling speed in response to 0.8 mM mianserin, similar to wild-type larvae (Supplementary Fig. 4a, Supplementary Table 3), demonstrating that the locomotor response to mianserin is not dependent on DA. In *C. elegans*, mianserin has been shown to target other monoaminergic pathways, including serotonergic neurotransmission.[65] To confirm that its effect on locomotion in larvae is mediated, as hypothesized, through the octopaminergic pathway, we fed mianserin to $Tdc2^{RO54}$ mutant larvae. We found that the crawling speed of these mutants, which lack OA, was unaltered, consistent with the behavioral activity of mianserin being mediated specifically through inhibition of OA activity (Supplementary Fig. 4a, Supplementary Table 3).

We next determined if using yeast suspended in mianserin solution as a vehicle to administer AMPH results in an enhanced fractional increase in speed in response to AMPH in wild-type, similar to that observed in *Tdc2* mutants. Larvae were fed yeast suspended in 0.8 mM mianserin solution containing increasing concentrations of AMPH, which led to a significant increase in crawling speed starting at 6 mM (16%, $P < 0.01$) (Fig. 2a). The hyperlocomotion was maximal at 60 mM AMPH (52%, $P < 0.001$) (Fig. 2a), which was chosen as the working concentration for all subsequent assays. We observed a larger fractional increase in velocity at all AMPH concentrations tested (Fig. 2a) compared to the increase observed at the same AMPH concentrations in the absence of mianserin (Fig. 1a).

In the presence of mianserin, as in its absence, the response to AMPH of larvae expressing TH-RNAi driven by TH-GAL4 (*TH-GAL4*/*UAS-TH-RNAi*) was blunted (Supplementary Fig. 4a, Supplementary Table 4), whereas control larvae carrying the non-driven TH-RNAi transgene (*UAS-TH-RNAi*/+) or the TH-GAL4 driver alone (*TH-GAL4*/+) responded to AMPH similarly to wild-type larvae (Supplementary Table 4). We also examined the locomotor behavior of larvae that lack TH, and thus DA biosynthesis, selectively in the nervous system ($DTHg^{FS\pm}$; *ple*).[49] These larvae did not display an increase in locomotion when fed AMPH whereas control larvae (*DTHg; ple*) exhibited a robust locomotor response to AMPH (Supplementary Fig. 4b, Supplementary Table 4), consistent with the data obtained using RNAi-mediated knockdown. In contrast, Tdc^{RO54} larvae displayed robust hyperlocomotion after feeding AMPH, and the addition of mianserin was without effect (Supplementary Fig. 4a, Supplementary Table 5). Taken together, these data establish that the AMPH-induced hyperlocomotion is strictly dependent on DA and that inhibiting OA

neurotransmission lowers the basal locomotor speed of larvae and leads to a larger fractional increase in speed in response to AMPH. For all subsequent assays, 0.8 mM mianserin was used as vehicle.

We then determined the impact of the duration of AMPH feeding. The hyperlocomotor response to 60 mM AMPH was significant after 30 min of feeding and persisted after 4 h of feeding (Fig. 2b). For all subsequent behavior assays, larvae were fed for 1 h to limit developmental changes during the timeframe of the experiment. Under these conditions, the behavioral response was the same over a 6 min period of data acquisition (Fig. 2c), and subsequent speed data were acquired for a period of 1 min after larvae were allowed 1 min of acclimation to the testing environment.

Amphetamine-induced hyperlocomotion is dependent on the dopamine transporter and its phosphorylation

AMPH leads to the release of DA into the extracellular space by inducing its efflux through DAT. To determine if DAT is essential for AMPH-induced behavior in *Drosophila*, we knocked down *Drosophila* DAT (dDAT) in DA neurons of larvae by targeted expression of dDAT-RNAi using the TH-GAL4 driver (*UAS-dDAT-RNAi/+*; *TH-GAL4/+*). Under conditions that elicited a robust behavioral response to AMPH in wild-type larvae as described above, depletion of dDAT diminished the response, indicating that the AMPH-induced hyperlocomotion is DAT-dependent (Fig. 3a, Supplementary Table 6). In contrast, control larvae expressing GFP-RNAi in DA neurons (*UAS-GFP-RNAi/TH-GAL4*) (Fig. 3a, Supplementary Table 6) or larvae carrying the non-driven dDAT-RNAi transgene (*UAS-dDAT-RNAi/+)* (Supplementary Table 6) responded to AMPH like wild-type. We also examined the locomotor response in larvae that carry a transposon insertion mutation of the *dDAT* gene (*dDAT^{fmn}*).[28] *dDAT^{fmn}* larvae did not display an increase in locomotion when fed AMPH, whereas the response was rescued upon expression of a human DAT transgene (*hDAT*) in the DA neurons of these mutant larvae using TH-GAL4 (*dDAT^{fmn}*; *UAS-hDAT/TH-GAL4*) (Fig. 3b, Supplementary Table 7). Control animals carrying the hDAT transgene or TH-GAL4 transgene alone in the *dDAT^{fmn}* mutant background (*dDAT^{fmn}; UAS-hDAT/+* or *dDAT^{fmn}; TH-GAL4/+*) did not show a significant response to AMPH (Fig. 3b, Supplementary Table 7). These findings established that DAT is essential for mediating hyperlocomotion in response to AMPH in *Drosophila* larvae.

Based on previous findings that phosphorylation of the DAT N-terminus is required for AMPH-induced DA efflux in heterologous cells[20, 21], we hypothesized that it is also required for AMPH-induced hyperlocomotion in larvae. To test this hypothesis we performed the above rescue experiment with two hDAT mutants: one in which the first five N-terminal serine residues were mutated simultaneously to alanines to prevent phosphorylation (hDAT-S/A), and one in which the same serines were mutated to aspartates to mimic their phosphorylated state (hDAT-S/D). In contrast to wild-type hDAT, expression of hDAT-S/A in DA neurons of *dDAT^{fmn}* larvae did not rescue the AMPH-induced response, whereas expression of hDAT-S/D resulted in robust hyperlocomotion in response to AMPH (Fig. 3b, Supplementary Table 7). These results (together with MPH data presented below) suggested that phosphorylation of the DAT N-terminus is necessary for

AMPH-induced behavior in larvae and that this assay can be used to investigate signaling mechanisms that modulate AMPH-induced changes in DAT function *in vivo*.

Methylphenidate induces DAT-dependent hyperlocomotion in *Drosophila* larvae

Unlike AMPH, MPH leads to elevated DA by competitively blocking the uptake of DA without inducing its efflux.[4] To determine if MPH can also elicit a behavioral response in larvae, we quantified the effect of acute MPH treatment on locomotion of wild-type larvae. Feeding w^{1118} larvae increasing concentrations of MPH for 1 h led to a significant increase in crawling speed starting at 2 mM MPH (13%, $P < 0.01$) (Fig. 4a). The hyperlocomotion was maximal at 3 mM MPH (19%, $P < 0.001$), which was chosen as the working concentration for all subsequent assays. Larvae expressing RNAi against *dDAT* in DA neurons (*UAS-dDAT-RNAi/+*; *TH-GAL4/+*) did not increase their crawling speed when fed MPH, indicating that this response is DAT-dependent (Fig. 4b, Supplementary Table 8). In contrast, larvae expressing control RNAi against GFP in the same neurons (*UAS-GFPi/TH-GAL4*) (Fig. 4b, Supplementary Table 8) or larvae carrying a non-driven dDAT-RNAi transgene (*UAS-dDAT-RNAi/+*) (Supplementary Table 8) responded to MPH like wild-type. Similarly, $dDAT^{fmn}$ mutant larvae, as well as control mutants carrying the UAS-hDAT transgene alone ($dDAT^{fmn}$; *UAS-hDAT/+*), exhibited no increase in speed when fed MPH (Fig. 4c, Supplementary Table 9), whereas expression of hDAT in DA neurons of $dDAT^{fmn}$ mutants ($dDAT^{fmn}$; *UAS-hDAT/TH-GAL4*) rescued the response to MPH. These data showed that DAT mediates the behavioral response to MPH in larvae, presumably by blocking reuptake of physiologically-released DA. Previous studies in heterologous cells have shown that inhibition of N-terminal phosphorylation of DAT has no significant effect on its uptake capacity.[20] In agreement with this finding, expression of either hDAT-S/A or hDAT-S/D rescued the MPH-induced hyperlocomotion (Fig. 4c, Supplementary Table 9), demonstrating both that these hDAT mutant constructs were functional and that the behavioral response to MPH does not require DAT phosphorylation.

Amphetamine-induced but not methylphenidate-induced hyperlocomotion is dependent on Flotillin-1

Recently we showed that the membrane raft protein Flot1 interacts in a complex with DAT and is necessary for DAT's localization to sterol- and sphingolipid-enriched membrane rafts.[22] However, neither knockdown of Flot1[22] nor disruption of lipid rafts by nystatin[22] or filipin[66] affects DA uptake by DAT in heterologous cells. In contrast, depletion of Flot1 severely blunts AMPH-induced DA efflux in mouse mesencephalic DA neurons in primary culture.[22] To investigate these findings at a behavioral level, we used the assay established above to determine whether loss of Flot1 function differentially affects the behaviors elicited by MPH (mediated by uptake inhibition) and AMPH (mediated by induction of efflux). The *Drosophila* genome codes for two Flotillin proteins (Flo and Flo-2), which are strongly expressed in neural tissues and localize to detergent-resistant membrane fractions in embryonic membranes, with Flo being the most closely related to the mammalian Flot1.[46, 67] We administered MPH to larvae that carry a transposon element insertion mutation of the *Flo* gene (Flo^{e02554})[47] (Supplementary Fig. 1). When Flo^{e02554} mutant larvae were fed MPH, they significantly increased their speed of locomotion (Fig. 5a, Supplementary Table 10). Similarly, RNAi-mediated depletion of Flo from the DA neurons

of larvae (*UAS-Flo-RNAi*/+; *TH-GAL4*/+) did not diminish their hyperlocomotive response to MPH (Fig. 5a, Supplementary Table 10). These data established that Flo is not required for DAT-mediated MPH-induced behavior. As the behavior elicited by MPH is mediated by inhibition of DA uptake, this indicated that the uptake function of DAT *in vivo* is not compromised by the loss of function of Flo, consistent with our findings in cultured cells. In contrast, since Flot1 is required for AMPH-induced DA efflux in mouse DA neurons in culture, we hypothesized that AMPH-induced hyperlocomotion in larvae is dependent on Flo. In contrast to treatment with MPH, treatment of Flo^{e02554} mutants with AMPH failed to increase their crawling speed (Fig. 5b, Supplementary Table 11). The behavioral response to AMPH was rescued by expressing human Flot1 (*hFlot1*) in the DA neurons of these mutants (*UAS-hFlot1*/+; Flo^{e02554}; *TH-GAL4*/+). These larvae displayed a robust increase in crawling speed when fed AMPH, in contrast to control Flo^{e02554} larvae that carry a non-driven hFlot1 transgene (*UAS-hFlot1/+*; Flo^{e02554}) (Fig. 5b, Supplementary Table 11). Similarly, larvae expressing RNAi against *Flo* driven by TH-GAL4 failed to respond to AMPH (*UAS-Flo-RNAi*/+; *TH-GAL4*/+) (Fig. 5c, Supplementary Table 12), whereas driving hFlot1 in addition to the Flo-RNAi transgene rescued the AMPH response (*UAS-hFlot1/+*; *UAS-Flo-RNAi*/+; *TH-GAL4*/+). In contrast, driving a palmitoylation-deficient mutant of hFlot1 (hFlot1C34A) that does not associate with the plasma membrane[22, 68] failed to rescue the response to AMPH that was blunted due to RNAi knockdown of *Flo* (*UAS-hFlot1C34A*/+; *UAS-Flo-RNAi*/+; *TH-GAL4*/+, Supplementary Table 12). Taken together, these data demonstrate that Flot1 in DA neurons is essential for the behavioral response to AMPH.

Discussion

Cocaine and MPH increase extracellular DA by blocking DAT-mediated reuptake of released DA.[2, 4] Other psychostimulants, such as AMPH, cause non-exocytotic release of DA from presynaptic nerve terminals through cytoplasmic release of DA from vesicles and subsequent efflux through DAT.[3] Since AMPH is a substrate for DAT, it has been hypothesized that it also exerts psychostimulant action through direct competition with DA[4], but it has been difficult to assess separately these two modes of psychostimulant action on DAT *in vivo* and thereby to evaluate their individual contributions to behavior. In this study we report a novel experimental model to investigate the molecular and neural mechanisms that underlie DAT-dependent behavioral responses to AMPH and MPH in *Drosophila*.

Our data show that larvae respond to acute doses of either AMPH or MPH by increasing their crawling velocity and that these responses are mediated by DA signaling. The hyperlocomotion response to AMPH is bigger than that to MPH, as expected given the difference in mechanisms of action of these drugs; AMPH's ability to deplete vesicular stores of DA and induce DA efflux likely leads to higher levels of synaptic DA.

Larvae with decreased levels of DA fail to exhibit an increase in speed in response to AMPH treatment. Furthermore, knockdown of dDAT by RNAi in TH-positive neurons or disruption of the *dDAT* gene blunts the response to both AMPH and MPH. Thus, the psychostimulant-induced locomotor response in larvae is DAT-dependent. hDAT can substitute functionally for dDAT, as selective expression of hDAT in DA neurons in an

otherwise *dDAT* null mutant background rescues the responses to both AMPH and MPH, suggesting that DAT-dependent psychostimulant-induced locomotor behavior is regulated by conserved mechanisms across species.

Selective expression of an N-terminal phospho-deficient hDAT mutant (hDAT-S/A) in DA neurons of larvae that lack functional dDAT rescues the locomotor response to MPH but not AMPH, consistent with previous findings in heterologous human embryonic kidney (HEK293) cells that this mutant can mediate DA uptake but fails to mediate AMPH-induced DA efflux.[20, 21] In contrast, larvae that express a mutant hDAT that mimics constitutive phosphorylation (hDAT-S/D) respond to AMPH, also consistent with the ability of hDAT-S/D to mediate AMPH-induced DA efflux in HEK293 cells. These cells lack VMAT, and thus DA accumulates in the cytoplasm and can efflux spontaneously via hDAT-S/D in the absence of AMPH at a supportive membrane potential[21]. However, AMPH-independent DA efflux should not occur in a neuron, as DA is sequestered inside vesicles and therefore not available for efflux. AMPH not only leads to redistribution of DA from vesicles into the cytoplasm but also increases intracellular Na^+, leading to depolarization, both of which enhance DA efflux.[69, 70] Therefore, while AMPH is not necessary to phosphorylate hDAT-S/D, it is still necessary for DA efflux, and therefore this “gain of function” mutation is still AMPH-dependent *in vivo*. Thus, while cell-based systems have provided important insights into the regulation of DAT, a comprehensive understanding of the mechanisms underlying AMPH action on DAT requires an *in vivo* system that expresses all the necessary components in a proper milieu where precise cellular and molecular manipulations can be performed and directly correlated with behavioral analyses.

We recently demonstrated that the membrane raft protein Flot1 is required for AMPH-induced efflux from mouse dopaminergic neurons in culture.[22] We have now demonstrated that Flot1 is essential in DA neurons for AMPH-induced behavior in *Drosophila* larvae. To our knowledge, this is the first evidence of a role for a membrane raft protein, or indeed of any DAT-interacting protein, in AMPH-induced behavior. Still, the precise mechanisms that underlie the role of Flot1 in AMPH-induced efflux and behavior remain to be determined. Flot1 interacts in a complex with DAT and is required to localize DAT in cholesterol-rich regions in the plasma membrane. Lipid rafts can scaffold key membrane-associated signaling events that are mediated by activated protein kinase C (PKC)[71-73] and Ca^{2+}/calmodulin-dependent protein kinases II (CaMKII)[74,75], both of which have been implicated in AMPH-induced DA efflux.[21, 76] Interestingly, we and others have recently observed that treatment of cultured cells with nystatin (Sen et al., manuscript in preparation) or filipin[66] to deplete cholesterol from membrane rafts inhibits PMA-induced phosphorylation of DAT. Taken together, our *in vivo* behavioral findings are consistent with a mechanistic model in which Flot1 promotes AMPH-induced DA efflux by localizing DAT to membrane microdomains where it can be phosphorylated by co-localized PKC and/or CaMKII. Using our novel assay, we can now test this model directly.

Despite its critical role in DA efflux, Flot1 is not necessary for DA uptake[22] and Flo is not required in larvae for MPH-induced behavior, consistent with the action of MPH as an uptake inhibitor. Thus, using our assay we can readily screen for and identify factors that specifically mediate behavior caused by AMPH-induced DA efflux rather than by inhibition

of DA reuptake. Moreover, because disruption of Flo completely ablates AMPH-induced hyperlocomotion, our results suggest that competition with DA is not a significant pharmacological contributor to this behavioral effect of AMPH.

In summary, the novel behavioral assay described in this manuscript, combined with the genetic tractability of *Drosophila*, provides an important tool to dissect DA signaling *in vivo*. It provides a rapid and efficient approach to probe directly the regulation of hDAT by kinases and other interacting proteins in response to psychostimulant treatment. Identified mechanisms can then be validated and more thoroughly investigated in mammalian models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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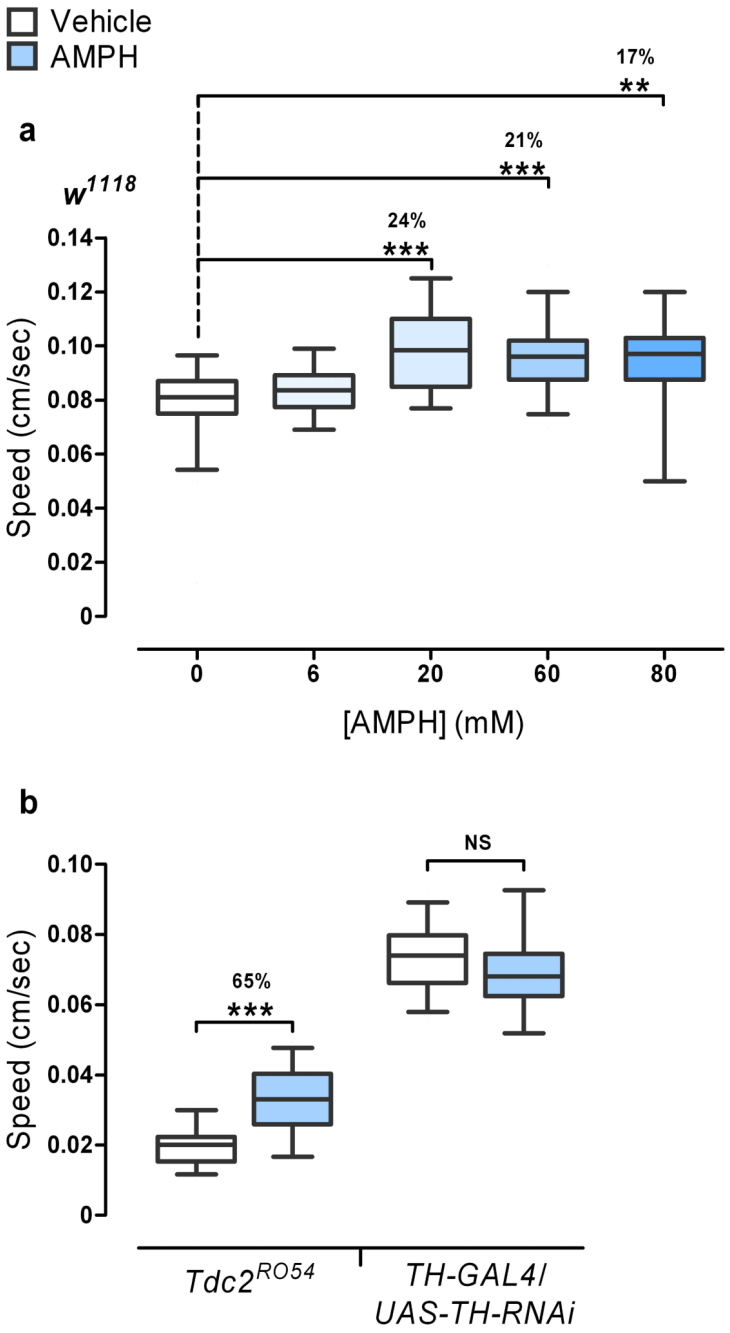

Figure 1. *Drosophila* larvae increase their crawling speed in response to acute AMPH treatment in a DA-dependent manner

(a) AMPH delivered in yeast suspended in water (vehicle) induced hyperlocomotion of wild-type (w^{1118}) larvae. AMPH at 20 mM, 60 mM and 80 mM led to a significant increase in velocity (24% [MWW test $U = 151$; ***$P < 0.001$], 21% [$U = 345.5$; ***$P < 0.001$], and 17% [$U = 90.50$; **$P < 0.01$], respectively). n=51 (0 mM), n=14 (6 mM), n=18 (20 mM), n=42 (60 mM), n=9 (80 mM). **(b)** Tdc^{RO54} fed 60 mM AMPH increased their crawling velocity by 65%. Larvae expressing RNAi against TH in DA neurons (*TH-GAL4/UAS-TH-RNAi*) did not respond to 60 mM AMPH. Boxplots represent the median as the middle line,

with the lower and upper edges of the boxes representing the 25% and 75% quartiles, respectively, and the whiskers representing the 5% and 95% quantiles. Asterisks indicate the significance of the increase in average crawling speed of larvae fed AMPH as compared to larvae of the same genotype fed vehicle (MWW test, NS $P > 0.05$, ***$P < 0.001$). Also see Supplementary Table 2.

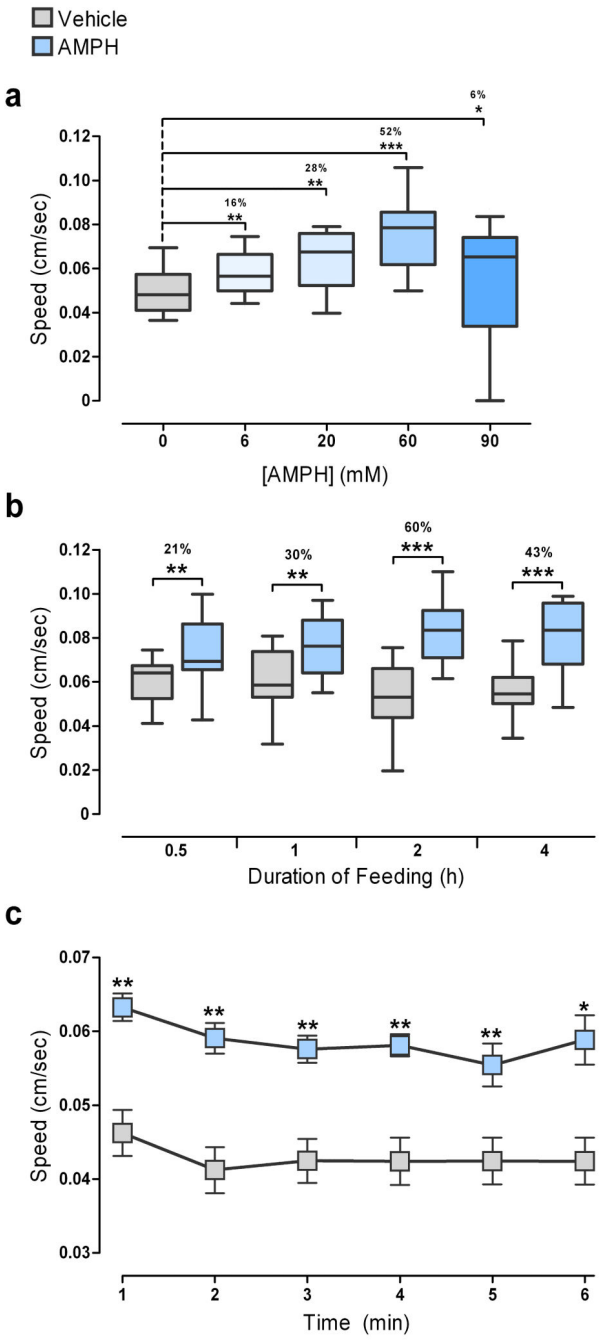

Figure 2. (a) *Drosophila* larvae display a larger fractional increase in speed in response to AMPH in the presence of mianserin

AMPH delivered in yeast suspended in 0.8 mM mianserin solution (vehicle) induced hyperlocomotion of wild-type (w^{1118}) larvae in a dose-dependent manner. AMPH at 6 mM, 20 mM, 60 mM and 90 mM led to a significant increase in velocity (16% [$U = 307$; $^{**}P < 0.01$], 28% [$U = 208$; $^{***}P < 0.001$], 52% [$U = 89.50$; $^{***}P < 0.001$] and 6% [$U = 280.5$; $^{**}P < 0.01$], respectively). n=36 (0 mM), n=30 (6 mM), n=30 (20 mM), n=30 (60 mM), n=26 (90 mM). (b) The response to AMPH persists with increasing durations of feeding. Wild-type larvae fed 60 mM AMPH for various lengths of time displayed a robust response

to AMPH. Larvae fed for 30 min, 1 h, 2 h and 4h increased their crawling speed by 21%, 30%, 60% and 43%, respectively, relative to control larvae fed vehicle for the same amount of time. Asterisks indicate the significance of the increase in average crawling speed in response to AMPH for the indicated feeding durations (**$P < 0.01$, ***$P < 0.001$). n=20/group. (c) The AMPH-induced hyperlocomotion in wild-type larvae persists over time. Wild-type larvae were fed vehicle or 60 mM AMPH for 1 h and their locomotion was recorded continuously over a period of 6 min. There was no significant effect of time of recording on the increase in speed of larvae fed AMPH. Wild-type larvae fed AMPH crawled 37% faster on average over the 6 min period compared to larvae fed vehicle. Asterisks indicate the significance of the increase in average crawling speed in response to AMPH for each minute (*$P < 0.05$; **$P < 0.01$). Speed was averaged over the 60 sec prior to each time point. n=10/group.

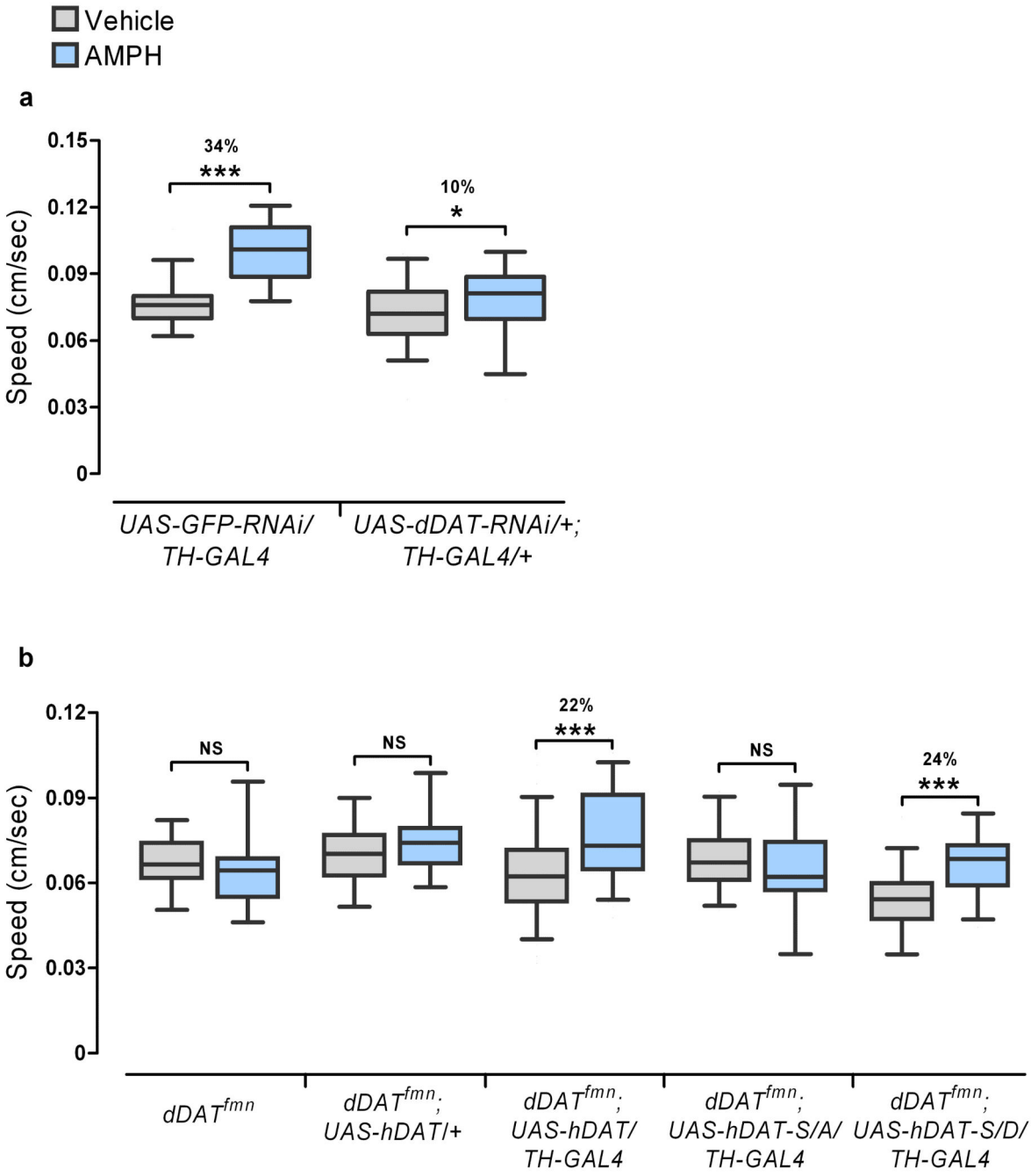

Figure 3. AMPH-induced hyperlocomotion is dependent on DAT and its phosphorylation

(a) RNAi-mediated knockdown of dDAT in DA neurons using the TH-GAL4 driver (*UAS-dDAT-RNAi*/+; *TH-GAL4/+*) blunted the response to AMPH as compared to expressing control RNAi against GFP in the same neurons (*UAS-GFP-RNAi*/*TH-GAL4*, 34% increase). Asterisks indicate the significance of the increase in average crawling speed of larvae fed AMPH as compared to larvae of the same genotype fed vehicle (*$P < 0.05$, ***$P < 0.001$). Also see Supplementary Table 6. **(b)** AMPH did not increase the speed of locomotion in *dDAT^{fmn}* larvae or in *dDAT^{fmn}* larvae carrying one copy of a non-driven hDAT transgene

(*dDAT*^{fmn}*; UAS-hDAT/+*). Expressing hDAT in DA neurons of *dDAT*^{fmn} larvae using the TH-GAL4 driver (*dDAT*^{fmn}*; TH-GAL4/UAS-hDAT*) restored the response to AMPH, as these larvae significantly increased their crawling speed by 22%. *dDAT*^{fmn} larvae expressing hDAT-S/A in DA neurons using TH-GAL4 driver (*dDAT*^{fmn}*; TH-GAL4/UAS-hDAT-S/A*) did not respond to AMPH whereas those expressing hDAT-S/D (*dDAT*^{fmn}*; TH-GAL4/UAS-hDAT-S/D*) responded by increasing their velocity by 24%. Asterisks indicate the significance of the increase in average crawling speed of larvae fed AMPH as compared to larvae of the same genotype fed vehicle (NS $P > 0.05$, ***$P < 0.001$). Also see Supplementary Table 7.

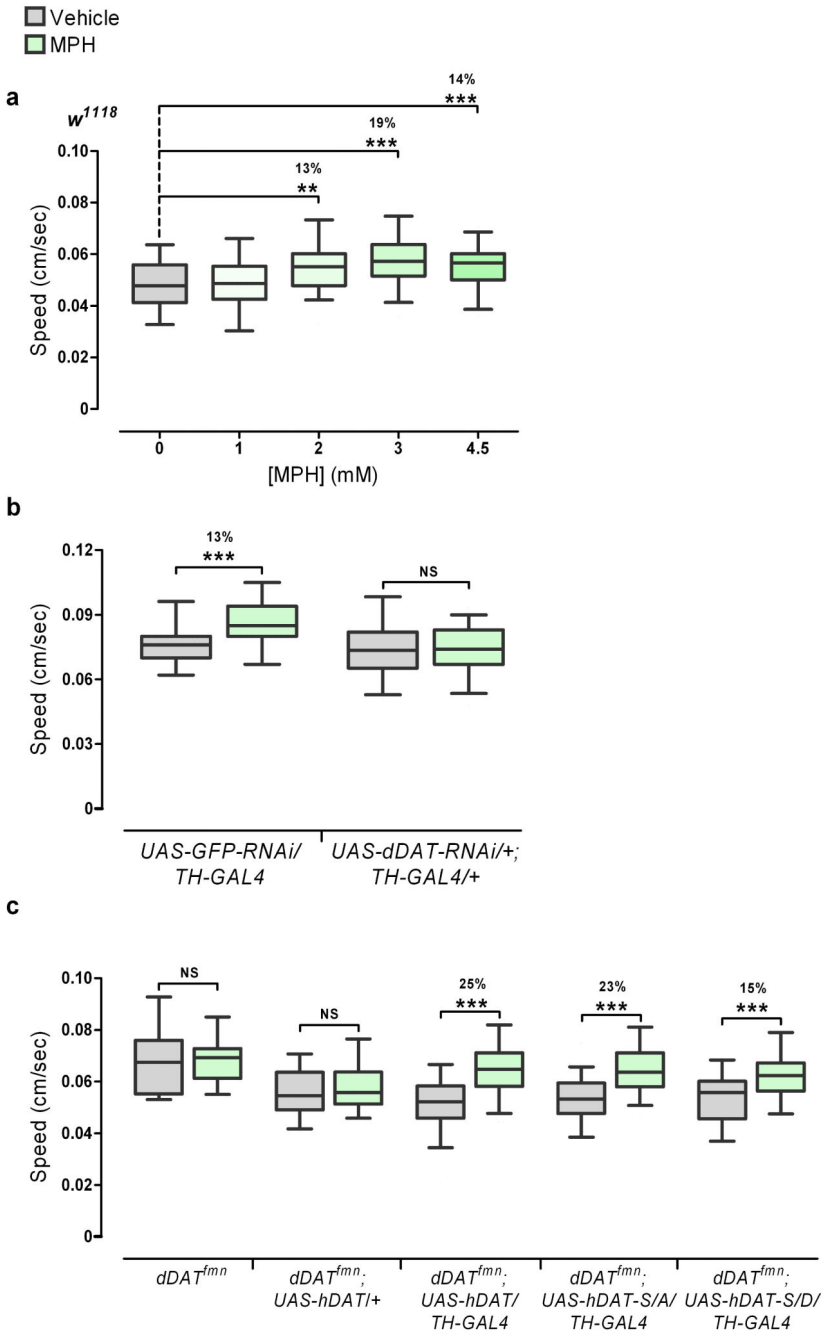

Figure 4. *Drosophila* larvae respond to MPH by hyperlocomotion in a DAT-dependent manner

(a) MPH induced hyperlocomotion of wild-type (w^{1118}) *Drosophila* larvae in a dose-dependent manner. MPH at 2 mM, 3 mM and 4.5 mM led to a significant increase in speed of locomotion (13% [$U = 565.5$; $^{**}P < 0.01$], 19% [$U = 460$; $^{***}P < 0.001$] and 14% [$U = 428$; $^{***}P < 0.001$] increase, respectively. n=44 (0 mM), n=40 (1 mM), n=41 (2 mM), n=45 (3 mM), n=36 (4.5 mM). **(b)** Larvae expressing RNAi against GFP in DA neurons (*UAS-GFP-RNAi*/*TH-GAL4*) responded to MPH by increasing their crawling speed by 13%. Expressing RNAi against dDAT in the same neurons (*UAS-dDAT-RNAi/+; TH-GAL4/+*)

blunted the response to MPH. Asterisks indicate the significance of the increase in average crawling speed of larvae fed MPH as compared to larvae of the same genotype fed vehicle (NS $P > 0.05$, ***$P < 0.001$). Also see Supplementary Table 8. **(c)** 3 mM MPH did not induce hyperlocomotion in *dDAT*^{fmn} mutants, or in *dDAT*^{fmn} mutants carrying one copy of a non-driven *hDAT* transgene (*dDAT*^{fmn}; *UAS-hDAT/+)*, whereas expressing hDAT, hDAT-S/A or hDAT-S/D in DA neurons of *dDAT*^{fmn} larvae using TH-GAL4 (*dDAT*^{fmn}; *UAS-hDAT/TH-GAL4*, *dDAT*^{fmn}; *UAS-hDAT-S/A/TH-GAL4*, or *dDAT*^{fmn}; *UAS-hDAT-S/D/TH-GAL4*) restored the response to MPH, as these larvae significantly increased their crawling speed by 25%, 23%, and 15%, respectively. Asterisks indicate the significance of the increase in average crawling speed of larvae fed MPH as compared to larvae of the same genotype fed vehicle (NS $P > 0.05$, ***$P < 0.001$). Also see Supplementary Table 9.

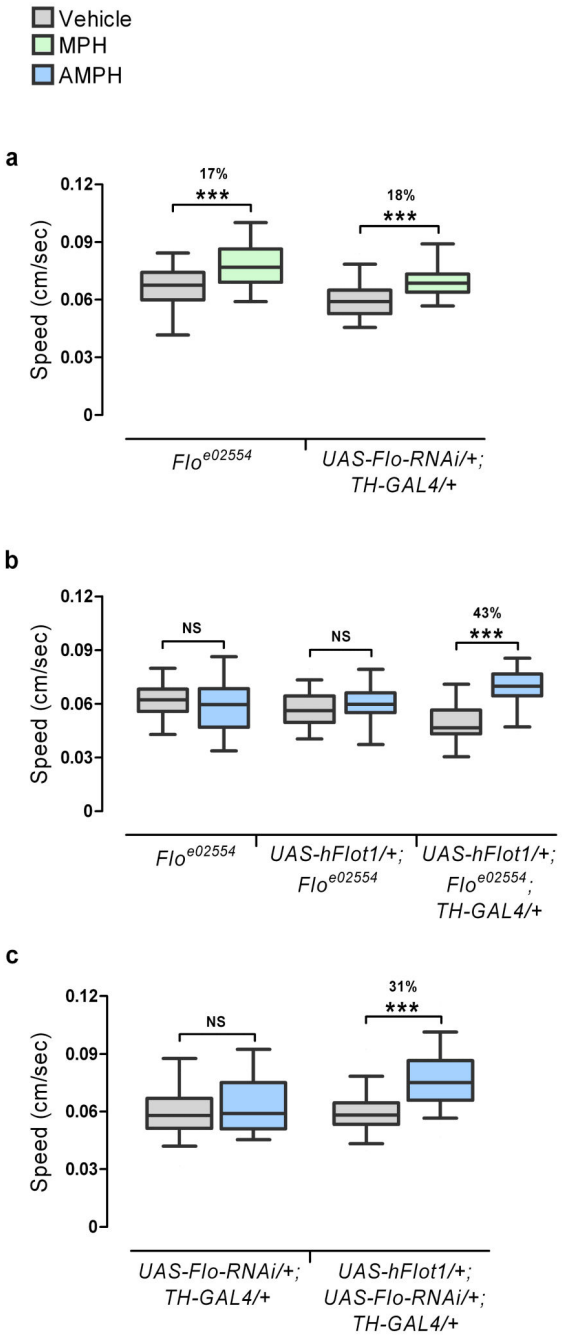

Figure 5. Flot1 is required for AMPH-induced but not MPH-induced hyperlocomotion in *Drosophila* larvae

(a) *Flo*^{e02554} mutant larvae and larvae expressing RNAi against Flo (*UAS-Flo-RNAi/+; TH-GAL4/+*) responded to MPH by increasing their speed by 17% and 18%, respectively. Asterisks indicate the significance of the increase in average crawling speed of larvae fed MPH as compared to larvae of the same genotype fed vehicle (***$P < 0.001$). Also see Supplementary Table 10. **(b)** AMPH did not significantly increase speed of locomotion in *Flo*^{e02554} mutant larvae or in *Flo*^{e02554} larvae carrying one copy of a non-driven *hFlot1*

transgene (*UAS-hFlot1/+*; *Flo*^{e02554}). In contrast, expressing hFlot1 driven by TH-GAL4 in DA neurons of *Flo*^{e02554} larvae (*UAS-hFlot1/+*; *Flo*^{e02554}; *TH-GAL4/+*) restored the response to AMPH as these larvae significantly increased their crawling speed by 43%. Asterisks indicate the significance of the increase in average crawling speed of larvae fed AMPH as compared to larvae of the same genotype fed vehicle (NS $P > 0.05$, ***$P < 0.001$). Also see Supplementary Table 11. **(c)** Larvae expressing Flo-RNAi in DA neurons (*UAS-Flo-RNAi/+*; *TH-GAL4/+*) did not increase their crawling speed in response to AMPH, whereas larvae expressing hFlot1 along with Flo-RNAi in DA neurons (*UAS-hFlot1*; *UAS-Flo-RNAi/+; TH-GAL4/+*) displayed a 31% increase in crawling speed. Asterisks indicate the significance of the increase in average crawling speed of larvae fed AMPH as compared to larvae of the same genotype fed vehicle (NS $P > 0.05$, ***$P < 0.001$). Also see Supplementary Table 12.