Molecular Mechanisms of Amphetamine Actions in Caenorhabditis elegans

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ABSTRACT

Amphetamine (AMPH) poses a serious hazard to public health. Defining the molecular targets of AMPH is essential to developing treatments for psychostimulant abuse. AMPH elicits its behavioral effects primarily by increasing extracellular dopamine (DA) levels through the reversal of the DA transporter (DAT) cycle and, as a consequence, altering DA signaling. In Caenorhabditis elegans, an excess of synaptic DA results in a loss of motility in water, termed swimming-induced paralysis (SWIP). Here we demonstrate that AMPH produces SWIP in a time- and dose-dependent manner in wild-type (wt) animals but has a reduced ability to generate SWIP in DAT knock out worms (dat-1). To determine whether D1-like and/or D2-like receptors are involved in AMPH-induced SWIP, we performed experiments in DOP-1 and DOP-4, and DOP-2, and DOP-3 receptor knockout animals, respectively. AMPH administration resulted in a reduced ability to induce SWIP in animals lacking DOP-3, DOP-4, and DOP-2 receptors. In contrast, in worms lacking DOP-1 receptors, AMPH-induced SWIP occurred at wt levels. Using microamperometry on C. elegans DA neurons, we determined that in contrast to wt cells, AMPH failed to promote DA efflux in dat-1 DA neurons. These data suggest that DA efflux is critical to sustaining SWIP behavior by signaling through DOP-3, DOP-4, and DOP-2. In a double mutant lacking both DAT-1 and DOP-1 expression, we found no ability of AMPH to induce SWIP or DA efflux. This result supports the paradigm that DA efflux through C. elegans DAT is required for AMPH-induced behaviors and does not require DOP-1 signaling.

Dysfunction in DA signaling has been implicated in multiple pathologies, including schizophrenia, attention-deficit/hyperactivity disorder, Parkinson’s disease, and AMPH abuse. Although it is well known that dopaminergic pathways are involved in cocaine and AMPH abuse, a comprehensive understanding of the key players coordinating AMPH behaviors is still missing. Upon exocytotic release of DA into the synapse, DATs coordinate the spatial and temporal action of DA by actively clearing the amine from the extracellular space. Although diffusion and enzymatic degradation may partially govern the synaptic concentration of DA, knockout studies have established DAT function as the primary mechanism controlling extracellular DA levels. DAT is the major molecular target responsible for the reward properties and abuse potential of several psychostimulants, including cocaine, methamphetamine, and AMPH. As a substrate of DAT, AMPH elevates extracellular DA levels by antagonizing DA clearance and stimulating DAT-mediated DA efflux. The subsequent increase in dopaminergic signaling mediated by DA receptors leads to secondary plasticity that mediates addiction. Whereas multiple lines of evidence implicate DA receptors in the mechanism of action of abused psychostimulants such as AMPH (Hiroi and White, 1991; Vallone et al., 2000; Ball et al., 2003), the degree to which different receptors support AMPH-induced behaviors has not yet been completely elucidated.

In Caenorhabditis elegans, DA is a prominent neurotransmitter synthesized in eight sensory neurons: two anterior deirid neurons, four cephalic neurons, and two posterior deirid neurons. In addition, three pairs of gender-specific dopaminergic neurons are present in the male tail (Sulston et al., 1975). The mammalian neuronal proteins involved in DA synthesis, vesicle loading, and reuptake all have C. elegans homologs, and mutants for each of these homologs have been described previously (Wintle and Van Tol, 2001; Nass and...
20 young adult worms were placed in 40 growth medium plates spread with NA22 bacteria. In each test, 8 to dopaminergic neurons. GFP under the control of the dat-1 promoter were used to identify amperometry experiments, transgenic animals expressing soluble DA has inhibitory effects on Blakely, 2003). Sawin et al. (2000) showed that exogenous DA biosynthesis and DA receptor signaling have been involved in changes in C. elegans locomotion in response to food exposure (Sawin et al., 2000; Chase et al., 2004). McDonald et al. (2007) identified a behavior mediated by DA termed swimming-induced paralysis (SWIP), which emerges when nematodes are placed in water, and is regulated by the DA transporter DAT-1.

Here, we took advantage of the nematode C. elegans to establish a model to elucidate the mechanism of AMPH action at DA synapses and in vivo subsequent behaviors. First, we show that AMPH treatment rapidly induces SWIP in wt but not DAT-1 knockout (dat-1) worms. In addition, we provide evidence that AMPH-induced SWIP requires DA synthesis, DA storage, and DA signaling. Finally, we demonstrate that cultured nematode DA neurons, like their mammalian counter parts, exhibit AMPH-induced DA efflux, which is required for SWIP. These results strongly suggest that DA released by AMPH through the transporter accounts for the behavior observed in animals treated with AMPH.

Materials and Methods

C. elegans Transgenic Animals. In this study, the wild-type strain used was N2 Bristol. All C. elegans strains were cultured on bacteria lawns of NA22 or OP50 and maintained at 20 °C using standard methods (Brenner, 1974). The dat-1(ok157III) strain was a gift from J. Duerr and J. Rand (Oklahoma Medical Research Foundation, Oklahoma City, OK), which is a complete loss-of-function mutation that eliminates the majority of the DAT-1 coding sequence. The dop-1(ut100X), dop-2(ut105V), dop-3(ut106I)X, dop-4(ok1321)X, cat-1(e1111)X, and cat-2(e1112)II were obtained from the Caenorhabditis Genetic Center (University of Minnesota, Minneapolis, MN). dat-1 dop-1 double mutants were created by crossing dat-1(ok157III) males with dop-1(ut100X) hermaphrodites and screened using single-worm polymerase chain reaction (Barstead et al., 1991; Williams et al., 1992) with oligonucleotides directed at dop-1(ok157III) and then further analyzed using oligonucleotides specific to dop-1(ut100X). Pdat-1::GFP; dop-3::GFP DAT-1 plasmid in the dop-3 strain. Animals positive for GFP fluorescence in DA neurons were selected. For amperometry experiments, transgenic animals expressing soluble GFP under the control of the dat-1 promoter were used to identify dopaminergic neurons.

SWIP Assay. Worms used for SWIP were grown on normal growth medium plates spread with NA22 bacteria. In each test, 8 to 20 young adult worms were placed in 40 µl of water with or without AMPH in a single well of a Pyrex spot plate (Thermo Fisher Scientific, Waltham, MA), and the number of paralyzed worms was reported as a percentage of the total number of animals observed in each test. At least 54 worms were tested per group. The number of paralyzed worms was counted every minute using an inverted microscope (Carl Zeiss, Inc., Thornwood, NY). Data were analyzed using Prism software (ver. 4; GraphPad Software, Inc., San Diego, CA).

Measurement of DA Efflux. DA neurons were isolated 2 days before recording from C. elegans embryos as described previously (Carvelli et al., 2004) and plated in glass-bottomed culture dishes (MatTech Corporation, Cincinnati, OH) coated previously with peanut lectin. Before recording, plates were washed twice with bath solution containing 145 mM NaCl, 5 mM KCl, 1 mM CaCl2, 5 mM MgCl2, 10 mM HEPES, and 20 mM d-glucose, pH 7.2 (325 osmolality adjusted with sucrose). DA fluxes were recorded using a carbon fiber electrodes connected to an Axopatch 200B amplifier ( Molecular Devices, Sunnyvale, CA), touching the plasma membrane of DA neurons expressing cytosolic GFP. Amperometric currents were digitized with an Axon Digidata 1320A, collected with pClamp 10.2, filtered with a low-pass Bessel filter set at 5 Hz, and analyzed using pClampfit 10.2 (all from Molecular Devices) and Origin 6.1 software (OriginLab Corp, Northampton, MA).

Results

AMPH-Induced SWIP Requires DAT-1 Expression. We have shown recently that an increase in endogenous extracellular DA levels triggers SWIP ( McDonald et al., 2007). Here, we tested whether the psychostimulant AMPH, which impedes and reverses DAT-1 function and, as a consequence, increases extracellular DA levels, promotes SWIP. Wild-type animals were placed into glass wells containing 40 µl of water (vehicle) with or without AMPH, and the emergence of SWIP was measured as a function of time. Under vehicle treatment, virtually no worms were paralyzed after 2 min, and after 4 min of energetic swimming, only 4 ± 2% of worms tested were paralyzed (Fig. 1A, solid symbols). At concentrations greater than 80 µM, AMPH produced significant SWIP at 4 min with respect to water-treated animals. Robust SWIP was achieved at 100 µM AMPH with 62 ± 12% of animals paralyzed after 4 min of AMPH treatment (Fig. 1A, solid symbols). At concentrations greater than 80 µM, AMPH produced significant SWIP at 4 min with respect to water-treated animals. Robust SWIP was achieved at 100 µM AMPH with 62 ± 12% of animals paralyzed after 4 min of AMPH treatment (Fig. 1A, solid symbols).

To verify whether DAT-1, which is expressed only in DA neurons ( Carvelli et al., 2004), is required for AMPH-induced SWIP, we used DAT-1 knockout animals (dat-1) in swimming assays in the presence and absence of AMPH. Figure 1B shows that 100 µM AMPH failed to produce statistically significant SWIP in dat-1 worms (○) with respect to vehicle-treated (■) worms (31 ± 5 versus 24 ± 8% of paralyzed
animals, respectively). However, because dat-1 mutants have a higher baseline of SWIP (McDonald et al., 2007) with respect to wt (compare Fig. 1, A, □, with B, ■), we focused on the AMPH-induced change in SWIP (ΔSWIP), which is calculated as the number of paralyzed worms upon AMPH treatment minus the number of paralyzed worms upon vehicle treatment. It is noteworthy that we found that AMPH-induced ΔSWIP is much higher in wt animals expressing DAT-1 than in dat-1 animals (Fig. 1B, inset). This supports the hypothesis that ablation of the dat-1 gene results in a decreased propensity of the animals to paralyze in water upon AMPH exposure and strongly supports the role of DAT-1 as a key player in AMPH-induced SWIP behavior.

AMPH-Induced SWIP Is Supported by DA Storage, DA Synthesis, and DA Receptor Signaling. DA signaling plays a central role in the behaviors induced by psychostimulants such as AMPH (Koob and Nestler, 1997). Therefore, to identify the key elements of dopaminergic signaling involved in AMPH-induced behaviors, we measured AMPH-induced SWIP in animals lacking DA biosynthesis (cat-2), DA packaging into vesicles (cat-1), and animals missing each of the four DA receptors (dop-1, 2, 3, 4). Figure 2A shows that cat-2 mutants, which are animals lacking tyrosine hydroxylase and have highly reduced DA levels (Sanyal et al., 2004), displayed significant reduction in AMPH effects compared with wt animals. At 4 min, in the absence of AMPH, these animals did not display basal SWIP (data not shown), possibly because of their highly reduced DA content.

It is well established that AMPH treatment results in a redistribution of DA from synaptic vesicles to the cytosol. Three models have been proposed to explain this phenomenon. First, in the weak base model, AMPH redistributes monoamines to the cytosol by collapsing the proton gradient required for vesicular accumulation of transmitter (Sulzer and Rayport, 1990; Rudnick and Wall, 1992; Sulzer et al., 1993), resulting in a reversal of vesicular monoamine transporters (VMATs). Second, as a substrate of VMAT, AMPH competitively inhibits DA uptake into vesicles, causing a leak of vesicular transmitter (Floor et al., 1995; Pothos et al., 2000). The third model suggests that the binding of nontransported AMPH to the cytosolic face of VMAT induces the release of transmitter from vesicles (Floor and Meng, 1996), although the mechanism of this phenomenon remains obscure. Considering that VMAT seems to play a fundamental role in these three models, we wanted to determine the requirement of VMAT in the mechanism of AMPH-induced SWIP by using cat-1 animals, which do not express VMAT (Sulston et al., 1975; Duerr et al., 1999). cat-1 animals did not show a significant change in basal SWIP with respect to wt animals (4.1 ± 2 versus 4 ± 2%). However, when we treated cat-1 mutants with AMPH, we measured significant reduction in ΔSWIP with respect to wt animals (Fig. 2B). These results suggest that a lack of DA accumulation into vesicles reduces the ability of AMPH to cause its behavioral effects, further supporting the role of VMAT in the AMPH actions.

To date, four DA receptors have been cloned and functionally characterized in C. elegans: the D1-like DA receptors Dop-1 and Dop-4 (Suo et al., 2002; Sugiuira et al., 2005); and the D2-like DA receptors Dop-2 and Dop-3 (Suo et al., 2003; Chase et al., 2004; Sugiuira et al., 2005). We investigated the involvement of these receptors in AMPH-induced SWIP using knockout lines for each receptor. Animals lacking Dop-1 (dop-1) did not differ from wt in their response to AMPH (Fig. 2C) or basal SWIP (data not shown). However, animals lacking Dop-2 (dop-2) showed a significant increase in basal SWIP compared with wt (34 ± 8% versus 4 ± 2%; p ≤ 0.02 by Student’s t test). It is noteworthy that in dop-2 animals, AMPH-induced ΔSWIP was significantly decreased with respect to wt (Fig. 2D). In Dop-3 and Dop-4 knockout animals (dop-3, dop-4), basal SWIP did not differ significantly from wt (data not shown); however, dop-3 and dop-4 animals displayed a robust decrease in AMPH-induced ΔSWIP (Fig. 2, E and F, respectively), achieving ΔSWIP levels similar to those measured in both cat-2 and dop-1 mutants. The 80% reduction in ΔSWIP detected in both dop-3 and dop-4 animals suggests that Dop-3 and Dop-4 receptors play a primary role in the transmission of DA signaling, which ultimately generates paralysis after AMPH treatment.

Although dop-1 animals respond to AMPH similarly to wt, it is possible that the decrease in ΔSWIP measured in dop-1 worms is mediated and/or requires Dop-1 signaling. To test this possibility, we generated the transporter/receptor double mutant line, lacking both DAT-1 and Dop-1 expression (dat-1 dop-1). Whereas AMPH-induced ΔSWIP in dop-1 animals showed a magnitude similar to that observed in wt animals (compare ■ in Fig. 3 with ● in Fig. 1A), the dat-1 dop-1 double mutants behaved as dat-1 mutants under vehicle treatment (compare △ in Fig. 3 with □ in Fig. 1B). After 4 min in water, 22 ± 7% of dat-1 dop-1 animals were paralyzed, and AMPH treatment did not significantly increase

![Fig. 2. AMPH-induced SWIP is supported by CAT-2, CAT-1, DOP-2, DOP-3, and DOP-4 expression. AMPH-induced ΔSWIP was significantly (Student’s t test) diminished in A animals lacking tyrosine hydroxylase (A; *p < 0.01; n = 6, 121 animals), VMAT (B; *p < 0.05; n = 11, 163 animals), DOP-2 (D; *p < 0.05; n = 14, 296 animals), DOP-3 (E; *p < 0.05; n = 8, 151 animals), and DOP-4 (F; *p < 0.005; n = 4, 106 animals) receptors compared with wt animals (dotted line). In contrast, AMPH-induced SWIP (C) was not significantly reduced in worms lacking DOP-1 (n = 12, 228 animals). All data are expressed as a percentage of wt.](molpharm.aspetjournals.org)
the percentage of paralyzed animals (23 ± 10%). Furthermore, ΔSWIP calculated for dat-1; dop-1 animals (Fig. 3, inset) was robustly reduced with respect to dop-1 animals. These data demonstrate that DOP-1 does not mediate the decrease in AMPH-induced ΔSWIP observed in dat-1 worms.

**SWIP Correlates with the Ability of AMPH to Induce DA Efflux.** It is known that AMPH treatment induces DA release through DAT, thereby altering the fidelity of DA signaling (Koob and Bloom, 1988; Di Chiara, 1995; Sulzer et al., 2005). Therefore, DAT has been hypothesized to be a key element in the basis of AMPH-induced behaviors (Giros et al., 1996; Sulzer et al., 2005). We sought to determine whether AMPH triggers DA efflux in *C. elegans* DA neurons and to establish whether efflux requires functional DAT. To this end, we created primary embryonic cultures of *C. elegans*, isolated dopaminergic neurons using a visual marker, and measured DA efflux by microamperometry. This technique takes advantage of a carbon-fiber electrode held at +700 mV, a voltage greater than the redox potential at which DA is oxidized. Thus, DA efflux stimulated by AMPH can be detected by the carbon fiber electrode as a measure of DA oxidation (Schroeder et al., 1994; Khoshbouei et al., 2003).

Primary neurons were obtained from embryonic cells of transgenic *C. elegans* worms carrying a transcriptional fusion of the -1 promoter fused upstream to the GFP gene. DA neurons were easily identified in these mixed cultures by GFP fluorescence. In DA neurons isolated from wt animals (which generate no redox signal on their own), 10 μM AMPH triggered DA efflux (0.07 ± 0.02 pA; n = 6), which was inhibited by 10 μM concentration of the DAT-1 inhibitor mazindol (−0.02 ± 0.003 pA; n = 6; Fig. 4A). AMPH-induced DA efflux was calculated subtracting the control amperometric currents (before AMPH) from the currents recorded in the presence of AMPH. AMPH-induced efflux measured in these cells was also inhibited by cocaine (data not shown). It is noteworthy that in DA neurons isolated from dat-1 animals, 10 μM AMPH failed to generate DA efflux (0.009 ± 0.007 pA, n = 5), and the application of 10 μM mazindol yielded no further effect (Fig. 4B). In DA neurons isolated from dat-1/dop-1 mutants, neither AMPH nor mazindol perfusion produced any effect in DA efflux (Fig. 4C), whereas in DA neurons isolated from dop-1 animals, which display AMPH-induced SWIP, perfusion of 10 μM AMPH induced DA efflux comparable with those measured in wt neurons (0.05 ± 0.03 pA, n = 4; Fig. 4D). Likewise, we measured DA efflux from dop-3 mutants containing the transcriptional fusion Pdat-1::GFP and bearing deletions that prevent AMPH-induced SWIP. No significant difference in AMPH-induced DA efflux was measured between dop-3 mutant (0.16 ± 0.07 pA, n = 3) and wt animals. These data reveal that the ability of AMPH to cause SWIP in *C. elegans* relies on both reverse transport of DAT and DA receptors signaling.

**Discussion**

To understand the long-term consequences induced by drugs of abuse, their initial molecular and cellular targets must be identified. A combination of approaches in animal models and humans has revealed the DA system to be the prime target of addictive drugs. Specifically, psychostimulants such as AMPH and cocaine bind to the DAT and alter its ability to clear DA from the synaptic cleft, thereby terminating the DA signal (Koob and Bloom, 1988; Nestler, 1992; Chen et al., 2006). In addition, AMPH-like drugs induce DAT to operate in reverse (Sulzer et al., 1993), therefore inducing nonvesicular DA efflux. Thus, the final result of AMPH is to elevate the extracellular concentration of DA and, as a consequence, stimulate DA signaling. Yet, the key molecular players involved in the behavioral effects induced by AMPH are still unclear. In particular, which subclass of DA receptors is triggered after AMPH treatment remains in question.

In *C. elegans*, DA plays at least two distinct roles: 1) it affects learning by mediating the state-dependent olfactory
adaptation (Colbert and Bargmann, 1995; Bettinger and McIntire, 2004); and 2) it modulates locomotor behaviors by slowing the locomotion rate when animals encounter bacteria (Sawin et al., 2000). Thus, in *C. elegans*, DA signaling responds to environmental changes by modulating locomotion, behavior, and learning. These studies relied primarily on the analysis of wt animals exposed to exogenous DA, ablation of DA neurons, and animals mutants for CAT-2, which are deficient in tyrosine hydroxylase activity (Loer and Kenyon, 1993; Liu and Sternberg, 1995; Schafer and Kenyon, 1995; Weinschenker et al., 1999; Sawin et al., 2000; Bettinger and McIntire, 2004; Hills et al., 2004; Sanyal et al., 2004).

McDonald et al. (2007) identified SWIP as a DAT-1-mediated behavior in *C. elegans*. This study took advantage of mutants lacking DAT-1 to show that under stress conditions, such as vigorous swimming, endogenous DA exerts an inhibitory action on motor circuits. However, wt animals, in which DAT-1 clears released DA from the synaptic cleft, do not paralyze. Here, we show that AMPH, which causes an increase in extracellular DA, induces SWIP. In addition, we reasoned that AMPH-induced SWIP might be a useful in vivo tool to determine the key elements of DA signaling sustaining the AMPH actions. Thus, by using *C. elegans* as a model system and SWIP as a behavioral test, we determined that AMPH specifically targets the DAT to promote DA release, and consequently, engages different classes of DA receptors, including DOP-2, DOP-3, and DOP4.

First, we show that in water, animals treated with different concentrations of AMPH paralyze in a dose- and time-dependent manner. In contrast, DAT-1 knockout animals had almost no response to AMPH treatment. It is noteworthy that DA neurons isolated from wt worms generated DA efflux upon AMPH stimulation, and this effect was blocked by the perfusion of DAT-1 inhibitors such as mazindol and cocaine. Consistent with the SWIP results, we were unable to measure a detectable DA efflux from neurons lacking DAT-1. These results suggest that DAT is a key element supporting the ability of AMPH to increase extracellular DA. Our data also support the hypothesis that increased levels of extracellular DA, released by AMPH and mediated by DAT-1, might be responsible for SWIP. The main role played by DAT-1 in DA transmission is revealed by the loss of AMPH-induced behaviors/efflux recorded in the DAT-1 knockout animals.

It is well known that DA can act on different DA receptor subtypes such as the D1- and D2-like receptors. These subtypes are believed to have different effects on forms of neuronal plasticity and, consequently, on learning and memory, because of their opposite action on the cAMP-dependent protein kinase A pathway (Missale et al., 1998; Cepeda and Levine, 1998; Floresco et al., 2001). Although DA and DA receptors are critically involved in AMPH-induced behaviors, the role of the various DA receptor subtypes has been difficult to delineate. For example, antagonizing D2-like receptors has been shown to attenuate the locomotor and rewarding effects of psychostimulants (Vallone et al., 2000; Ball et al., 2003). However, the role of D2-like receptors in the molecular mechanisms of psychostimulant abuse is not fully understood. In rodents, D2-like receptor activation decreases AMPH-induced locomotor activity and induces no change in AMPH-induced efflux in the ventral tegmental area (Stecketee and Kalivas, 1992). Thus, we reasoned that by studying the role of the different DA receptors in AMPH-induced behaviors in a simple organism such as *C. elegans*, it would allow us to define whether they play a role in the AMPH actions. In this study, we have shown that animals lacking DOP-2, DOP-3, or DOP-4 receptors have a decreased ability to paralyze upon AMPH treatment. In contrast, dop-1 animals still respond to AMPH treatment. Moreover, our data demonstrate that AMPH-induced SWIP requires endogenous DA. Only a very small percentage of animals lacking the tyrosine hydroxylase enzyme are paralyzed after AMPH treatment. Therefore, our results strongly suggest that DA, DAT-1, and the DA receptors DOP-2, DOP-3, and DOP-4 are key players in AMPH-induced behaviors. Our data also support that *dat-1* deletion does not require changes in DOP-1 signaling to decrease AMPH-induced SWIP because *dat-1; dop-1* animals show robustly reduced ∆SWIP.

These data also identify, for the first time, DOP-4 as an important player in *C. elegans* motor behavior. DOP-4 receptors have been shown to localize in GABA AVL motoneurons, the PQR chemosensory neurons, and other head neurons (Sugiura et al., 2005). Although we do not know whether the *c. elegans* DA neurons make synapses with neurons expressing DOP-4 receptors, we cannot exclude the possibility that the release of large amounts of extracellular DA after AMPH treatment may lead to spillover of the neurotransmitter to extrasynaptic sites. Curiously, worms lacking VMAT (*cat-1* animals) also showed significant reduction of ∆SWIP compared with wild-type animals. Thus, it seemed that the greater amount of DA released after AMPH application in wt animals is also due, in part, to the action of AMPH on VMAT (Jones et al., 1998; Sulzer et al., 2005). However, although *cat-1* animals demonstrated only a 50% reduction of ∆SWIP, *dat-1, cat-1, dop-3*, and *dop-4* animals displayed more dramatic reductions in AMPH response.

Furthermore, we have shown that after AMPH treatment, the increase in extracellular DA targets DOP-2, DOP-3, and DOP-4 signaling to generate SWIP. In addition, SWIP is sustained both by vesicular storage of DA and DA synthesis. Forward genetic screens are unbiased but extremely costly and time-consuming in mammalian models. Thus, we propose to implement forward genetic screens to study the actions of AMPH using *C. elegans*. The treatment of intact worms with AMPH leads to a DAT and DA receptor-dependent phenotype termed SWIP, which we propose as a reporter phenotype for a powerful, unbiased screen to identify the genes required for AMPH action. Therefore, the future goals of this research will be to identify suppressors of AMPH-induced SWIP, followed by focused secondary screens that establish a presynaptic locus for identified mutants and their DA and DAT relationship.

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