Caenorhabditis elegans as an in vivo Model to Assess Amphetamine Tolerance

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Keywords

Amphetamine · Tolerance · *Caenorhabditis elegans*

Abstract

Amphetamine is a potent psychostimulant also used to treat attention deficit/hyperactivity disorder and narcolepsy. In vivo and in vitro data have demonstrated that amphetamine increases the amount of extra synaptic dopamine by both inhibiting reuptake and promoting efflux of dopamine through the dopamine transporter. Previous studies have shown that chronic use of amphetamine causes tolerance to the drug. Thus, since the molecular mechanisms underlying tolerance to amphetamine are still unknown, an animal model to identify the neurochemical mechanisms associated with drug tolerance is greatly needed. Here we took advantage of a unique behavior caused by amphetamine in *Caenorhabditis elegans* to investigate whether this simple, but powerful, genetic model develops tolerance following repeated exposure to amphetamine. We found that at least 3 treatments with 0.5 mM amphetamine were necessary to see a reduction in the amphetamine-induced behavior and, thus, to promote tolerance. Moreover, we found that, after intervals of 60/90 minutes between treatments, animals

were more likely to exhibit tolerance than animals that underwent 10-minute intervals between treatments. Taken together, our results show that *C. elegans* is a suitable system to study tolerance to drugs of abuse such as amphetamines.

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Introduction

Psychostimulants, such as amphetamines and cocaine as well as all addictive drugs, significantly increase the amount of dopamine released by the dopaminergic neurons in the brain's reward centers [Di Chiara and Imperato, 1988]. Several groups have shown that hyperactivity of the dopaminergic neurons, with a consequent increase in dopamine levels, represents the first step that ultimately generates addiction [Volkow et al., 1999; Sulzer, 2011]. Specifically, psychostimulants directly bind to and alter the function of dopaminergic proteins such as the dopamine transporter [Mortensen and Amara, 2003; Zhu and Reith, 2008] and the vesicular monoamine transporter (VMAT) [Partilla et al., 2006; Freyberg et al., 2016]. In fact, in mice lacking expression of the dopamine transporter or expressing a cocaine-insensitive dopamine

transporter, cocaine had reduced or completely lost reinforcing effects, respectively [Thomsen et al., 2009a, b].

Before and while drug addiction occurs, people develop tolerance to the drug. This means that the effects generated by these drugs are diminished after repeated use and, for this reason, higher doses of drug are needed to achieve the initial effects [Ambre et al., 1988; Calipari et al., 2014]. This process can rapidly escalate to drug overdose. Though few studies have investigated the neurobiological mechanisms of tolerance in relation to the dopaminergic response to psychostimulants [Barnett et al., 1987; Mateo et al., 2005], some studies reported that, while mice with intermittent access to cocaine exhibited sensitization of the dopaminergic response, i.e., increased release and uptake of dopamine [Calipari et al., 2013], continuous access to cocaine for 6 h generated tolerance as measured by a decrease in cocaine-induced locomotion [Ferris et al., 2013]. However, whether dopamine is involved in the mechanisms that ultimately generate drug tolerance has not been proven yet. One limiting factor has been the absence of a drug-induced phenotype that is exclusively mediated by dopamine. While murine models offer several advantages in studying the effects generated by psychostimulants [Müller, 2018; Golden et al., 2019], behaviors in mammals are often the result of the action of multiple proteins. For this reason, it is not an easy task to dissect a drug-induced behavior into its molecular components in mammals. On the other hand, invertebrate models, because of their higher degree of simplicity, have been instrumental in studying the cause-effect relation between genes/proteins and behaviors [Søvik and Barron, 2013; Engleman et al., 2016; van Staaden and Huber, 2019]. For example, the nematode *Caenorhabditis elegans* (*C. elegans*) is a broadly used model for genetic dissection of animal behaviors. With only 302 neurons, *C. elegans* is capable of performing a variety of behaviors including motor and learning behaviors [Hobert, 2003; Ardiel and Rankin, 2010]. Thus, we reasoned that *C. elegans* might help us to identify the genes/proteins involved in the mechanisms that ultimately generate tolerance to amphetamine. The use of *C. elegans* is also justified by the fact that most of the proteins linked to the dopaminergic neurons, which are the main targets of psychostimulants, are highly conserved in mammals and *C. elegans* [Lai et al., 2000]. For example, proteins involved in the synthesis, storage, release, and reuptake of dopamine, as well as the D1- and D2-like dopamine receptors, share a high degree of structural and functional similarities between *C. elegans* and humans [Chase and Koelle, 2007]. Moreover, we and other

groups have shown that the *C. elegans* dopamine transporter (DAT-1), the vesicular monoamine transporter (CAT-1), and the D2-like dopamine receptor (DOP-3) exhibit pharmacological profiles similar to those observed in mammals [Nass et al., 2002; Carvelli et al., 2004, 2008, 2010; McDonald et al., 2007; Refai and Blakely, 2019]. More specifically, we demonstrated that, as in mammals, DAT-1 inhibitors inhibit dopamine uptake [Carvelli et al., 2004], and amphetamine induces dopamine efflux through DAT-1 in cultures of dopaminergic neurons isolated from *C. elegans* embryos [Hossain et al., 2014; Safratowich et al., 2014]. Importantly for this study, we have previously shown that *C. elegans* exhibits a unique behavior when treated with amphetamine. While *C. elegans* swim vigorously in water, in the presence of amphetamine they gradually stop moving and sink to the bottom of the well. We named this behavior swimming-induced paralysis (SWIP). Using both genetic and pharmacological approaches, we demonstrated that amphetamine-induced SWIP is caused by an overload of dopamine in the synapses which, in turn, overstimulates the D2 post-synaptically located receptors DOP-3, causing reversible paralysis in the worms [Carvelli et al., 2010]. Indeed, when the medium is replaced with amphetamine-free solution, animals repristinate their ability to swim. The excess of extracellular dopamine can be achieved by treating wild-type animals with dopamine releasers such as amphetamine [Carvelli et al., 2010] or by blocking the activity of the dopamine transporter either pharmacologically [Carvelli et al., 2008] or genetically [McDonald et al., 2007; Carvelli et al., 2010]. In both cases, we observe SWIP. Importantly, genetic ablation of tyrosine hydroxylase, the key enzyme for dopamine synthesis, prevents SWIP induced by a lack of function of DAT-1 [McDonald et al., 2007], confirming, therefore, dopamine as the main culprit of the SWIP behavior.

Since the data mentioned above have already demonstrated that amphetamine causes a consistent and quantifiable behavior (SWIP) in *C. elegans*, and that SWIP is mainly mediated by overflow of extracellular dopamine [McDonald et al., 2007; Carvelli et al., 2010], we investigated whether *C. elegans* develops tolerance to amphetamine by using SWIP as a behavioral readout. By repeatedly exposing *C. elegans* to amphetamine and testing them for SWIP, we can not only see if the behavioral response to amphetamine changes over time but also correlate such changes to the dopaminergic activity since SWIP is predominantly caused by overstimulation of the DOP-3 receptors through the endogenously released dopamine.

Fig. 1. Amphetamine (AMPH)-induced behaviors decrease after multiple exposures to the drug. Animals were immersed in a solution containing the control (open squares) or the control and 0.5 mM AMPH (open circles) for 10 min. While no change in the percentage of animals exhibiting SWIP was observed between the first

exposure (a) and the second exposure (after 30 min) (b) to AMPH, a strong reduction was observed during the third exposure (after 60 min) (c) ($p < 0.001$) and the fourth exposure (after 90 min) (d) ($p < 0.001$) with respect to the first exposure (two-way ANOVA). Cont, control.

Materials and Methods

C. elegans Husbandry and Age Synchronization

Both wild-type (N2) and *dat-1* knockout animals (*dat-1(ok157)*) were obtained from the Caenorhabditis Genetic Center (University of Minnesota) and grown at 20 °C in agar plates seeded with NA22 bacteria. The animals underwent age synchronization to ensure that all animals tested were at the same age [Kudumala et al., 2019]. Briefly, gravid adult animals were collected from plates using autoclaved Milli-Q water, transferred into a 15-mL conical tube, and pelleted by centrifugation at 1,200 rpm for 3 minutes (min). The supernatant was removed using a sterile tip connected to vacuum aspirator and Milli-Q water was added to the tube to resuspend the pellet. This step was repeated at least 2 times or until the supernatant was clear from bacteria. In order to release the embryos, worms were lysed with a mixture of 1.0 mL of 3% NaOCl, 0.25 mL 10 N NaOH, and 3.75 mL water for 5–10 min or until 70% of the animals were lysed. The tube was filled with egg buffer (in mM: 118 NaCl, 48 KCl, 2 CaCl₂·2H₂O, 2 MgCl₂·6H₂O, 25 HEPES, pH 7.3, and 335–346 mOsm/L) and centrifuged at 1,200 rpm for 3 min to form a pellet. Then, the supernatant was removed from the tube and the pellet was resuspended in egg buffer. This

step was repeated 3 times to wash off any residual bleach. The pellet was then suspended in 30% sucrose solution (5 mL of water and 5 mL of 60% sucrose), and the tube was centrifuged at 1,200 rpm for 6 min to separate the embryos from the debris in the sucrose gradient. Embryos were then collected at the solution meniscus using a sterile glass pipette and transferred into a 15-mL conical tube. The tube was filled to 10 mL with autoclaved water and centrifuged at 1,200 rpm for 3 min. This step was repeated a total of 3 times to remove sucrose from the samples. After the last centrifugation, the supernatant was removed and M9 buffer (for 1 L of buffer: in g, 3 KH₂PO₄, 6 NaHPO₄, 5 NaCl, and 1 mL of 1 M MgSO₄) was added to the tube, which was then centrifuged, and once again filled with M9 solution to 10 mL. The tube containing the embryos suspended in the M9 solution was kept for 14 h at room temperature on a shaker. During this time, all embryos will mature into the first larval stage and they will not grow beyond this stage because the M9 medium does not contain food. This guarantees age synchronization of all of the animals. Larvae were washed with water, placed on agar plates containing food (NA22 bacteria), and kept for about 36 hours in a 20 °C incubator or until the animals reached the late-L4 larval stage.

Fig. 2. Aging does not account for the decreased behavioral effects seen after repeated exposure to amphetamine (AMPH). **a** The percentage of animals exhibiting SWIP over a 10-min treatment with the control solution (open squares) or AMPH (open circles) is reported during the fourth exposure. Animals that received 3 consecutive exposures to the control solution followed by 1 AMPH exposure (closed circles) exhibited higher levels of SWIP with respect to animals that were exposed 4 times to AMPH (* $p < 0.001$; two-way ANOVA). **b** The percentage of animals exhibiting SWIP after a 10-min treatment with AMPH during the first, second, third, and fourth exposures is shown. Values collected after the

third and fourth exposures to AMPH were statistically different from values collected during the first and second exposures (* $p < 0.005$ and * $p < 0.0005$ for 4th AMPH exp vs. 1st AMPH exp and vs. 2nd AMPH, respectively). Animals that were treated 3 times with the control solution and 1 time with AMPH exhibited statistically higher levels of SWIP than animals treated 4 times with AMPH ($\infty p < 0.05$; one-way ANOVA). However, no difference was measured between animals treated 1 time with AMPH and those treated 3 times with the control solution and 1 time with AMPH (compare first and last bars). The number of animals tested per each group are as in **b**. Cont, control; exp, exposure.

Behavioral Assay (SWIP)

SWIP was performed as previously published [Kudumala et al., 2019]. Briefly, under a dissecting microscope, 8–12 late-L4 larvae were transferred with an eyelash pick from the plate to a glass spot plate well containing 40 μ L of 0.5 mM amphetamine dissolved in 200 mM sucrose solution. Sucrose solution was used instead of milli-Q H_2O to guarantee constant osmolarity (200 mOsm/L). The animals were left in the amphetamine solution for 10 min and, every minute, the number of paralyzed animals was recorded to quantify the percent of animals exhibiting SWIP. After 10 min, the animals were transferred into a well containing 40 μ L of water for about 1 min. Then, the animals were transferred to an NA22 bacteria-containing plate and allowed to recover for 30 min. The animals were transferred from the recovery plate into another glass well containing fresh amphetamine solution and then tested for 10 min again for SWIP, washed off in water, and allowed to recover for 60 min on the agar plate. This procedure was done one more time, but a 90-min recovery time was allowed before the animals were tested again for amphetamine-induced SWIP. Parallel experiments were performed using 200 mM sucrose solution alone (control group). To prevent contamination between the amphetamine- and control-treated groups, we thoroughly washed the eyelash pick in ethanol and water while we transferred the animals from and to the wells.

Rigor and Statistical Analysis

Each SWIP assay was performed using 8–12 animals per group. For the experiments shown in Figure 1, a total of 35 trials were performed on 15 different days over a period of 5 weeks and a total of 263 or 115 animals were repeatedly exposed to amphetamine or control solutions, respectively.

The data shown in Figure 2a includes a total of 614 animals (246 treated 3 times with the control and 1 time with amphetamine, 253 treated 4 times with amphetamine, and 115 treated with the control 4 times). The animals were tested in 22 separate trials in 11 days over a 5-month period.

The experiments shown in Figure 2b reflect the same numbers of animals presented in Figures 1 and 2a.

The experiments shown in Figure 3a include the 22 trials shown in Figure 2a plus 6 extra trials for a total of 28 trials and 129 animals treated 4 times with the control solution, 299 animals treated 4 times with amphetamine, and 300 animals treated 3 times with the control solution and 1 time with amphetamine solution.

The experiments shown in Figure 3b were performed in 4 trials and included 39 animals exposed 2 times to the control solution and 1 time to the amphetamine solution, 40 animals exposed to amphetamine 3 times and 28 animals exposed to the control solution 3 times.

Statistical analysis was performed with GraphPad Prism software using one-way ANOVA (Fig. 2b) or two-way ANOVA (Fig. 1a–d, 2a, 3a, b) with a Bonferroni multiple comparison post-test. The error bars in Figures 1–3 represent SE.

Fig. 3. Tolerance to amphetamine (AMPH) is maintained over 40-min treatments and with reduced intervals between exposures. **a** Animals were exposed to the control solution (open squares), AMPH (open circles), or the control solution followed by AMPH (closed circles) as described in Figure 2a. However, during the last exposure, the percentage of animals exhibiting SWIP was measured up to 40 min. The percentage of animals exhibiting SWIP in the group that received 4 AMPH exposures was statistically lower than the animals that received 3 exposures to the control solution and 1 exposure to AMPH throughout the 40-min observation

(* $p < 0.001$; two-way ANOVA). **b** Animals were exposed 3 times to the control solution (open squares) or the AMPH solution (open circles) and 2 times to the control solution followed by 1 exposure to AMPH (closed circles). Between each exposure, the animals were allowed to recover and feed in drug-free agar plates for 10 min. A statistical difference between the animals that received 3 AMPH exposures and those that received 2 control exposures followed by 1 AMPH exposure was achieved after 6 min of the last exposure (* $p < 0.05$; two-way ANOVA). Cont, control; exp, exposure.

Results

Repeated Exposure to Amphetamine Reduces Amphetamine-Induced Behaviors

To investigate whether *C. elegans* develop tolerance to amphetamine, we measured amphetamine-induced SWIP in wild-type animals during and after repeated exposure to the drug (Fig. 1). The animals had 4 consecutive 10-min exposures to amphetamine with in-between recoveries of 30, 60, and 90 min on agar plates seeded with food. Longer intervals between treatments were used to allow animals to feed and recover from swimming while undergoing treatments. During the first exposure, among the animals immersed in a solution containing 0.5 mM amphetamine, there was a gradual increase in the number of paralyzed animals (SWIP) and this reached a plateau at 10 min (open circles in Fig. 1a). After 10 min, $70 \pm 5\%$ of the animals exhibited SWIP (white bar in Fig. 2b). Parallel experiments showed that animals exposed to the control solution did not exhibit SWIP over the 10-min timeframe (open squares in Fig. 1a). These results confirm our previously published data showing that amphetamine causes SWIP in *C. elegans* [Carvelli et al., 2010; Safratowich et al.,

2013; Lanzo et al., 2018]. Following the first exposure to the amphetamine or control solution, the animals were reallocated to food-seeded plates after being briefly immersed (~ 1 min) in wells containing water to ensure that no residue of amphetamine was left on the animals' bodies. After 30 min on the plate, the animals were subjected to a second treatment with amphetamine and, after a 10-min exposure, $76 \pm 4\%$ of the animals exhibited SWIP (open circles in Fig. 1b and 2nd AMPH exp bar in Fig. 2b). Again, the animals were briefly washed in water and transferred to the plate, where they recovered and fed for 60 min before being exposed to amphetamine for the third time. During the third exposure, we measured a significant reduction in the number of animals exhibiting SWIP over the 10-min exposure (open circles in Fig. 1c). Specifically, after 10 min, $42 \pm 6\%$ of the animals exhibited SWIP (3rd AMPH exp bar in Fig. 2a; $p < 0.05$ for the third AMPH exposure vs. the second amphetamine exposure). Finally, the animals were exposed for the fourth time to amphetamine after a recovery period of 90 min in the plate (open circles in Fig. 1d). We observed a statistically significant reduction in SWIP with respect to the first and second exposures (compare Fig. 1a, d, and 2b), with only

27 ± 9% of the animals exhibiting SWIP after 10 min (4th AMPH exp bar in Fig. 2b; $p < 0.005$ and $p < 0.0005$ for the fourth amphetamine exposure vs. the first amphetamine exposure and vs. the second amphetamine exposure, respectively). Similar to the first exposure, no animal exhibited SWIP during the second, third, and fourth exposures to the control solution (open squares in Fig. 1b–d). These results show that, while 2 amphetamine exposures caused no change in how the animals respond to the drug, 3 or more exposures caused a significant reduction in the behavioral outcomes induced by amphetamine.

Repeated treatment with amphetamine caused a reduction in the percentage of animals exhibiting SWIP (open circles in Fig. 1c, d), but no change in SWIP was observed in animals that were repeatedly treated with the control solution (open squares in Fig. 1c, d). Thus, we could conclude that recurring exposure to amphetamine is the sine qua non cause of the reduced SWIP observed in Figure 1c, d. We cannot exclude, however, that a reduced behavioral response to amphetamine may occur in *C. elegans* because of an additive effect of aging, i.e., animals could respond differently to amphetamine as they age. As a matter of fact, between the first and last exposures to amphetamine, there was an interval of 4 hours which, for an animal like *C. elegans* with a lifespan of 2–3 weeks, could represent a significant aging step. Moreover, repeated swimming assays could per se alter the response to amphetamine. To exclude these possibilities, we designed another set of experiments done in parallel to the experiments described above, where a group of animals, after being exposed 3 times to the control solution, was treated with amphetamine for the first time (closed circles in Fig. 2a). In this group a larger number of animals exhibited SWIP with respect to the group that was exposed to amphetamine 4 consecutive times (open circles in Fig. 2a; $p < 0.0001$; two-way ANOVA). Importantly, after 10 min, the percentage of SWIP in the animals that received 3 control exposures followed by 1 amphetamine exposure was not statistically different from the percentage in those exposed to amphetamine for the first time (i.e., 56 ± 11 and 70 ± 5%, respectively; compare the gray and white bars in Fig. 2b). However, a significant difference was measured between this group and animals that received 4 amphetamine treatments (compare gray and 4th AMPH exp bars in Fig. 2b; $p < 0.05$). These data demonstrate that aging or repeated swimming assays do not account for the reduced response to amphetamine observed after repeated treatment.

Next, we tested whether the reduced behavioral response between the first and third treatments was maintained if the last amphetamine treatment was prolonged

for up to 40 min. Animals were treated with 0.5 mM amphetamine or the control solution for 10 min during the first, second, and third exposures. During the last exposure, the animals underwent the fourth treatment with amphetamine or the control solution for 40 min (open circles and open squares, respectively, in Fig. 3a). Likewise, the subgroup of animals that received three 10-min treatments with the control solution were then treated with amphetamine for 40 min (closed circles in Fig. 3a). As shown in Figure 3a, the significant difference between the percentage of animals treated 4 times with amphetamine (open circles) and those treated 3 times with the control solution followed by 1 amphetamine treatment (closed circles) was maintained for up to 40 min ($p < 0.001$; two-way ANOVA).

The data shown above were collected during treatments with amphetamine or the control solution at intervals spanning from 30 to 90 min. Since no difference was observed after the first interval of 30 min between the first and second exposures (Fig. 1a, b), we tested whether a shorter interval between treatments would change the outcomes seen in Figure 1. Thus, animals were treated with amphetamine 3 times with in-between intervals of 10 min (Fig. 3b). Again, we measured a statistically significant reduction of SWIP in animals that received 3 amphetamine treatments (open circles in Fig. 3b) with respect to the group that received 2 control solution treatments and 1 amphetamine treatment (closed circles in Fig. 3b; $p < 0.05$; two-way ANOVA). However, while during longer intervals (30–90 min) between treatments (Fig. 2a) the statistical significance between the 2 groups was achieved after 3 min, using 10 min intervals between treatments statistical significance was achieved after 6 min. Moreover, the power of the analysis was stronger in trials with longer in-between intervals (60–90 min) than in those with 10-min intervals ($p < 0.001$ and $p < 0.05$, respectively). Taken together, these results show that 3 or more amphetamine exposures reduce the ability of *C. elegans* to exhibit SWIP. Thus, they suggest that *C. elegans* develops tolerance to amphetamine. Moreover, they suggest that 60/90-min intervals between the second/third and third/fourth treatments are more likely to generate tolerance to amphetamine than are 10-min intervals.

Discussion

Living organisms have developed self-regulating processes that are used to keep their biological functions to a set point with a relatively narrow range of operation. This

self-regulating process is known as homeostasis. When external/internal perturbations occur, animals respond to the change by activating different mechanisms aimed to reestablish the original homeostatic equilibrium. Based on this concept, tolerance to a drug might be proposed as a process of adaptation to the perturbing effect of the drug [Peper et al., 1987, 1988]. As drug tolerance is revealed through a slow decline in the effect of the drug after repeated administrations, this might suggest that the body slowly learns how to counteract the effects produced by the drug such that the overall system can run within its operation set point.

Although tolerance is a phenomenon that can occur with any type of drug, it is particularly important to understand the mechanisms that generate tolerance to drugs of abuse because, due to their addictive nature, these drugs are remarkably dangerous. In fact, while the “well-being” effects (euphoria, relaxation, and arousal) dissipate as tolerance takes place, the toxic effects caused by addictive drugs after prolonged use at high concentrations build up. Moreover, because drugs of abuse affect and alter the reward system, when tolerance to drugs of abuse occurs, people feel the urge for more drug. Escalation of drug use can ultimately lead to death.

Different types of addictive drugs exist, but one common feature among all of them is their ability to activate, either directly or indirectly, the dopaminergic system. Indeed, previous data suggest that overflow of extracellular dopamine is the first step leading to addiction [Sulzer, 2011]. However, it is unknown whether dopamine plays a role in the process that generates drug tolerance. Here we have investigated tolerance to amphetamine because this drug directly interacts with specific proteins of the dopaminergic system and because amphetamine is broadly used and abused as a psychostimulant. To be able to investigate the molecular mechanisms underlying the development of tolerance to amphetamine, it is necessary to use a model organism that can develop tolerance to this drug and, at the same time, allows straightforward assays at the molecular level. We decided to consider *C. elegans* as a model to study the development of tolerance to amphetamine mainly because (1) the simplicity of the nervous system and the genetic tools this nematode offers allow us to directly correlate specific behaviors to genes/proteins. This same approach would be very tedious, if not impossible, in mammalian models. (2) The dopaminergic synapse is highly conserved between *C. elegans* and mammals. (3) Since *C. elegans* exhibits the amphetamine-induced behavior SWIP, we can use SWIP to test whether repeated exposures to amphetamine would di-

minish the behavioral response and, thus, we can test whether amphetamine causes tolerance in *C. elegans*. (4) Finally, because SWIP requires mainly dopaminergic proteins, we can test whether dopamine plays a role in amphetamine tolerance.

Our results indicate that, when *C. elegans* is repetitively exposed to amphetamine, a reduction in the amphetamine-induced behavioral response (SWIP) occurs. This suggests that *C. elegans* develops tolerance to amphetamine. The animals that were repeatedly exposed to the control solution did not show SWIP, excluding therefore the possibility that worms were paralyzed due to fatigue from being forced to swim. At least 3 exposures to the drugs were necessary to see tolerance to amphetamine (Fig. 1) and the inclusion of age-matched control groups allowed us to rule out the possibility that the decreased amphetamine-induced SWIP was caused by aging (Fig. 2). As a matter of fact, the animals exposed 4 times to the control solution did not show SWIP, whereas animals exposed 3 times to the control solution and the fourth time to the amphetamine solution showed higher levels of SWIP than the group exposed to amphetamine 4 times.

Someone could argue that the reduction of a dopamine-dependent behavior (SWIP) after repeated amphetamine treatments is the result of dopamine depletion from vesicles. If this is true, then longer time intervals between treatments allow more time for replenishment of dopamine stores via tyrosine hydroxylase activity. Consequently, we should see a stronger reduction of SWIP during short intervals between amphetamine treatments but reduced effect or no effect during longer recovery intervals. However, this scenario is not supported by our data. In fact, animals were more likely to develop tolerance when 60- or 90-min intervals (~140 or 240 min in total from the first exposure) were allowed between treatments with respect to 10-min intervals (~100 min in total from the first exposure). In addition, our experiments showed that the behavioral effects caused by repeated exposure to amphetamine were maintained for up to 40 min during the fourth exposure (280 min in total from the first exposure), suggesting that it is unlikely that diminished dopamine store levels are responsible for the observed tolerance to amphetamine. However, because no data about the turnover of the *C. elegans* dopamine vesicle are currently available, more experiments need to be done to establish the role of dopamine depletion in the amphetamine-induced tolerance seen in our behavior experiments. This notwithstanding, our results demonstrate that repeated exposure to amphetamine reduces amphetamine-induced behavior and suggests that *C. elegans* de-

velops tolerance to amphetamine. Accordingly, *C. elegans* may be used as a model organism to study the underlying molecular mechanisms of tolerance caused by repeated exposure to psychostimulants.

Two main types of tolerance to drugs exist, i.e., acute and chronic. Acute or short-term tolerance occurs within 1 session of consecutive drug administration (binges) during which the effectiveness of the drug decreases after the first dose. This is a transient effect that does not carry over into other sessions performed on different days [Hatsukami et al., 1994]. For example, in humans the increased heart rate, elevated blood pressure, and euphoric effects caused by the first dose of cocaine cannot be attained 40 min later or even after nearly doubling the concentration of cocaine in the blood [Ambre et al., 1988; Foltin and Haney, 2004]. In contrast, in chronic or long-term tolerance, the decreased effectiveness of a drug persists over time, during different sessions performed on different days, and it is likely a form of neuronal adaptation to constant exposure to a drug for months or years [Kalant et al., 1971].

The acute tolerance caused by psychostimulants in pseudo drug-naïve subjects resolves by itself within a relative short period. Thus, we could assume that psychostimulants temporarily and reversibly alter the activity of specific protein targets and, if these proteins are responsible for the acute physiological/behavioral effects caused by psychostimulants, then consecutive exposures to the drugs would result in reduced physiological/behavioral outcomes. Alternatively, the proteins activated by psychostimulants and responsible for the acute effects induced by these drugs could be a separate set of proteins/mechanism from those generating acute tolerance. The two processes, however, must be somehow connected since a reduced effectiveness of a drug (tolerance) implies dampened physiological/behavioral effects.

In the case of chronic tolerance, the reduced effectiveness of the drugs after recurrent and long-term use of psychostimulants could be due to permanent alteration of the proteins/mechanisms underlying the acute tolerance phenomena. Then again, different proteins/mechanisms, that were not affected during acute tolerance, might be recruited when chronic tolerance takes place. Regardless of whether acute and chronic tolerance share the same type of proteins/mechanisms or not, the first step to understand drug tolerance is identification of the proteins/mechanisms underlying this phenomenon. This is a challenging task because of the complexity of the mammalian nervous system. On the other hand, the neuronal circuits in *C. elegans* are small and well defined, thus making the

correlation between gene activity within single cells and the behavior of a mutant phenotype a much less intimidating task than in mammals.

Here, we have shown that *C. elegans* after repeated treatments develop tolerance to amphetamine. As the life cycle of *C. elegans* is very short (3–4 days) compared to those of mammals, and because in *C. elegans* tolerance to amphetamine is maintained over time after the third and fourth exposures (Fig. 3a), we speculate that the amphetamine-induced tolerance observed in our experimental paradigm is a type of chronic tolerance. In fact, as observed in humans treated with cocaine, *C. elegans* showed a dampened response to amphetamine only after multiple 10-min treatments (Fig. 1). As the SWIP assay has been previously characterized as a predominantly dopamine-mediated phenotype [McDonald et al., 2007; Carvelli et al., 2010], we can speculate that the reduced response to amphetamine observed in our experiments involves a reduced activity of one of the proteins responsible for the SWIP phenotype, i.e., (1) tyrosine hydroxylase (which is the key enzyme in the dopamine synthesis), (2) the vesicular monoamine transporter (which stores dopamine into the synaptic vesicles), (3) the dopamine transporter (which reuptakes dopamine into the neurons and mediates dopamine efflux), and (4) the D2 receptors. These 4 proteins are required by amphetamine to generate SWIP in *C. elegans* [Carvelli et al., 2010]. Future experiments targeting each of these proteins will identify which of these proteins is required to initiate tolerance.

In summary, the data presented here demonstrate that *C. elegans* develops tolerance to amphetamine. Thus, *C. elegans* can be used as model to identify the molecular mechanisms underlying tolerance. Moreover, as genetic screens in *C. elegans* are well established and commonly used to assess gene function in any biological process, the behavioral paradigm presented in this study can be used to identify the genes/proteins involved with amphetamine tolerance.

Statement of Ethics

This study did not include human subjects or vertebrate animals.

Conflict of Interest Statement

The authors have no conflict of interests to declare.

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Author Contributions

L.C. and S.K. designed the experiments. D.T.V., S.K., and M.H. performed the experiments. L.C. analyzed the results and wrote this paper.

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