Stereotypic behavioral responses to free-base cocaine and the development of behavioral sensitization in *Drosophila*

Colleen McClung and Jay Hirsh

Cocaine abuse is a large social and economic problem that has received much public and scientific attention in recent years. Rodent and primate models have been used to study the behavioral and neurological effects of cocaine. Repeated intermittent doses of cocaine lead to progressive increases in both locomotor activity and stereotyped behaviors known as 'reverse tolerance' or behavioral sensitization, which may model the behavioral and neurochemical processes occurring in cocaine-addicted humans [1]. The biological basis of sensitization is poorly understood. We report that free-base cocaine administered in volatile form to the fruit fly *Drosophila melanogaster* induces multiple reflexive motor responses that resemble cocaine-induced behaviors in rodents. These behaviors are both dose dependent and sexually dimorphic. Furthermore, *Drosophila* develops a behavioral sensitization to intermittent doses of cocaine. These results suggest that the pathways leading to cocaine-induced responses and sensitization are evolutionarily conserved between *Drosophila* and higher vertebrates, and that this genetically tractable animal can be used as a new model system to help determine the biological mechanisms underlying these processes.

Address: Department of Biology, University of Virginia, Charlottesville, Virginia 22903, USA.

Correspondence: Jay Hirsh
E-mail: jh6u@virginia.edu

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Results and discussion

To determine whether *Drosophila* show behavioral responses to cocaine, flies were exposed to cocaine free-base volatilized from a heated filament (see Materials and methods), then transferred to a viewing chamber where behaviors were assessed following videotaping of the flies. We find flies show behavioral responses following exposures to cocaine vapors that are strikingly similar to the reflexive motor behaviors observed in rodents [2,3]. Furthermore we note many similarities between the cocaine-induced behaviors in these intact flies and behaviors that can be induced by biogenic amines or dopamine agonists in the nerve cord of decapitated flies [4]. The cocaine-induced behaviors progress in a specific order from mild responses of intense grooming to the most severe — paralysis or sometimes death — in a dose-dependent manner. We have devised a behavioral scoring system based on these observed behaviors. The behavioral scores range from 0 to 7, where 0 represents normal behavior and 7 is the most severe (Table 1). As a control for specificity, flies were exposed to 100 μg of the (+)-isomer of cocaine, which is >100 times less active than (–)-cocaine in vertebrates [5]. This exposure leads to no discernible behavioral phenotypes (data not shown), indicating that the induced behaviors do not result from non-specific irritation from the cocaine aerosol.

Table 1

Behavioral scoring of flies following exposure to cocaine free-base vapors.

0	Normal behavior: locomotion, flight with a basal level of grooming.
1	Intense nearly continuous grooming and reduced locomotion.
2	Stereotyped locomotion, extended proboscis. Some locomotion with simultaneous grooming. In this and higher behavioral scores there is a loss of negative geotaxis and flight, with flies remaining at the bottom of the container.
3	Slow stereotypic locomotion in a circular pattern, extended proboscis.
4	Rapid twirling, sideways or backwards locomotion sometimes accompanied by a front leg twitch.
5	Hyperkinetic behaviors including bouts of rapid rotation, wing buzzing, erratic activity with flies often bouncing off the wall of the container.
6	Severe whole body tremor, no locomotion, usually overturned with legs contracted to body.
7	Total akinesia or dead.

The cocaine-induced behaviors are dose dependent. Flies exposed to ≤ 25 μg cocaine show no abnormal behaviors (Behavior 0), whereas all flies exposed to ≥ 200 μg cocaine show akinesia and tremors or death (Behaviors 6–7). Doses of 75–100 μg of cocaine induce the widest array of behavioral responses. To determine responses in this range of doses, flies were exposed to cocaine for 1 minute then transferred to a viewing chamber and videotaped for 5 minutes. The median values for the highest behavioral scores achieved by around 20 flies in each treatment group exposed to 75 μg or 100 μg cocaine during the first and last minutes of the 5 minute testing interval are shown in Table 2. By comparing median values of the behavioral scores, 100 μg cocaine produces a more severe response than 75 μg, and all groups except for females exposed to 75 μg show significant recovery over the interval studied.

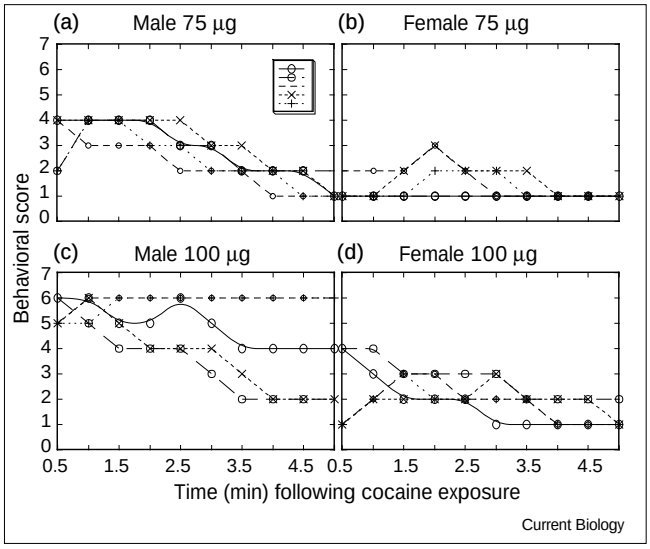
Table 2

Median behavioral scores following cocaine exposure.

Group	Time (min)	Group behavioral scores: Median	Lower quartile	Upper quartile	*P values
75 µg M	0–1	4	3	4	$P = 0.002$
	4–5	2	1	2.5	
75 µg F	0–1	1	1	1.5	$P = 0.16$
	4–5	1	1	1	
100 µg M	0–1	5	4	5.5	$P = 0.0001$
	4–5	2	1.5	4	
100 µg F	0–1	2	1	3	$P = 0.022$
	4–5	1	1	2	

*Median values from the 0–1 and 4–5 min intervals were compared with the Wilcoxon Rank Sum procedure ($P < 0.0253$). Significant P values are in bold. Highly significant differences ($P \leq 0.002$) are also found when comparing the 0–1 min time points as a function of sex (M/F) or amount of cocaine. For each group, $n = 20 \pm 3$ flies.

Figure 1



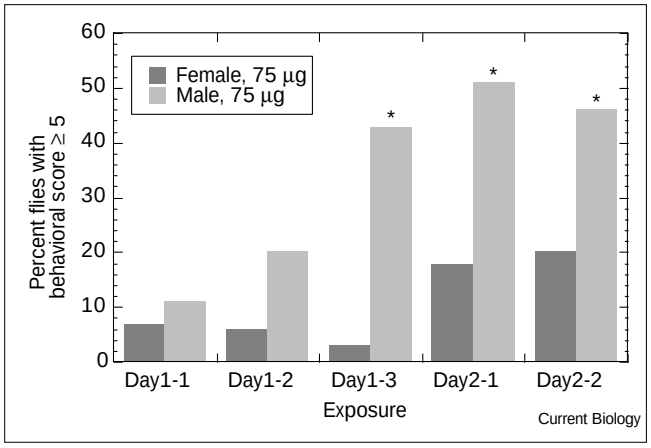
Behavioral responses to cocaine free-base vapors as a function of time following exposure, doses of cocaine and sex. Flies were exposed to 75 µg (a,b) or 100 µg (c,d) volatilized free-base cocaine. Responses of individual flies are shown separately for males (a,c) or females (b,d). Flies were exposed to cocaine at time –1 to 0 min, transferred to the viewing chamber at time 0 and the first score recorded at 0.5 min. Behavioral scores shown are the scores observed for individual flies at 30 sec observation intervals during a 5 min time period. The distinct symbols represent different individual flies picked at random for observation.

Following exposure to cocaine, the flies progress rapidly to their most severe behavioral response and then display the behaviors in descending order during the recovery period. To show the stepwise progression of behavioral responses over the period subsequent to cocaine exposure, we plotted the behavioral responses of five individual males and females chosen at random from treatment groups exposed to 75 µg (Figure 1a,b) or 100 µg (Figure 1c,d) of cocaine (Figure 1). The progression of behaviors to the most severe response is very rapid, generally taking between 30–150 seconds from the beginning of the 1 minute cocaine exposure. Often this upward progression of behaviors occurs during the exposure to cocaine, before flies are placed in the viewing chamber. The progression of behaviors in the recovery phase is slower, with flies returning to normal behaviors within 5–10 minutes after a moderate dose of cocaine. These results are consistent with the rather short half-life of cocaine in vertebrates [6].

Also apparent from Table 2 and Figure 1 is that the behaviors elicited by cocaine are sexually dimorphic, in that males are more severely affected than females. Similarly, decapitated *Drosophila* show sexually dimorphic responses to the D2-like dopamine agonist quinpirole when it is applied to the nerve cord, with males showing greater sensitivity than females [4]. Although males are smaller than females, we do not think that this is a factor. When genetically small flies are tested, their behavioral responses are not significantly different from those of wild-type flies (data not shown). Vertebrate models also show sexually dimorphic responses to psychomotor stimulants; female rodents are more sensitive to cocaine and amphetamine than are males [3,7,8]. This increased sensitivity has been linked to the higher estrogen levels present in females [9,10]. The sexual dimorphism observed in *Drosophila* cannot be due to sex hormones such as estrogen or testosterone as flies do not contain these hormones. Interestingly, when ovariectomized female rats and castrated male rats are given amphetamine, females display less stereotypy than do males [11], suggesting that there may be a hormone-independent sex difference in rodents that parallels what we find in *Drosophila*.

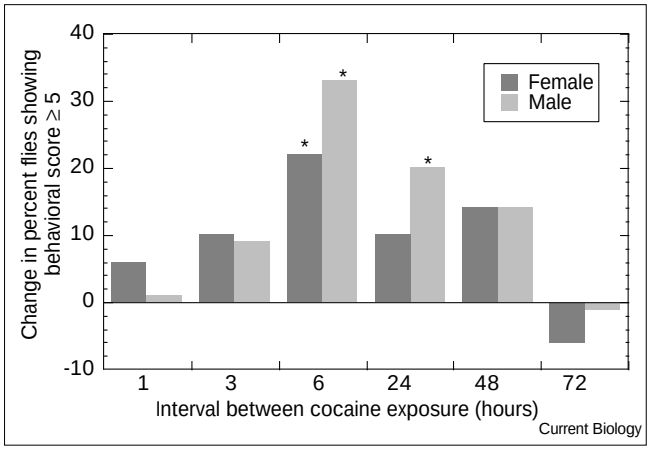
Vertebrate models show enhanced responsiveness to intermittent repeated exposures to cocaine [12,13]. To determine if *Drosophila* show similar sensitized responses, flies were given three 75 µg doses of cocaine at 3 hour intervals between doses, followed by two exposures the following day with 3 hours between doses, for a total of five exposures to cocaine over 2 days. Behavioral responses were scored after each exposure. Males develop a significant sensitization to cocaine by the third exposure, which continues through the following day (Figure 2). This shows that sensitization persists for at least 18 hours, as males are still maximally sensitized at the first dose on the second day of treatment. Females fail to develop a significant sensitization under this treatment paradigm, although, as shown below, this is due to the reduced responsiveness relative to males at the dose used.

Figure 2



Sex-dependent sensitization to intermittent cocaine exposures. Flies were exposed to 75 μg of cocaine three times, with 3 hour intervals between doses, on day 1. They were then given two exposures of cocaine with 3 hour intervals between exposures on the following day. The percentage of flies showing a behavioral response ≥ 5 during a 5 min testing period is shown for each exposure. 30–35 flies were assayed for each data point. Significant sensitization compared to the initial exposure is denoted by the asterisks as determined by chi-squared analysis ($P < 0.01$).

Figure 3



Sensitization to cocaine as a function of interval between doses. Flies were exposed twice to cocaine at intervals as shown. The ordinate shows the change in percentage of flies showing behavioral scores of ≥ 5 at any time in a 5 min interval following cocaine exposure, comparing the second minus the first cocaine treatment for each group of flies. Male flies were exposed to 75 μg, and females were exposed to 110 μg cocaine per vial, with 75–80 flies assayed for each data point. Chi-squared analysis indicates that flies of each sex show significant variation during their second cocaine exposure as a function of interval between exposures ($P \leq 0.025$). Statistically significant differences ($P \leq 0.009$, chi-squared) for flies of individual sex and time interval compared to the same groups of flies during their first cocaine exposure are shown by asterisks. As controls, flies were given an initial 'sham' treatment followed by a cocaine exposure to ensure that sensitization was not due to effects of handling. These flies showed no increased behavioral responses over flies that did not undergo the sham treatment (data not shown).

Rodents can show sensitization following even a single exposure to cocaine [14]. To determine if *Drosophila* show a similar response, and to examine whether sensitization is dependent upon the time interval between exposures, *Drosophila* were exposed to a single dose of cocaine and then to a subsequent dose at an interval of 1 to 72 hours later. Males were exposed to 75 μg cocaine and females were given 110 μg, doses that produce equivalent initial behavioral responses. We find a significant sensitization in both sexes, with maximal sensitization occurring with a time interval of 6 hours between doses (Figure 3). Significant sensitization is first detectable at 6 hour intervals, and persists at significant levels for intervals of 24 hours. Furthermore, significant sensitization is not observed when flies are given two cocaine doses at intervals of 48–72 hours. These results show that sensitization is highly dependent upon the time interval between doses. Moreover, these results show that the changes occurring in the brain in response to a single cocaine dose are long lasting in terms of the 2–3 week life span of *Drosophila*, but are not permanent.

Two models could explain the observed sensitization to repeated cocaine exposures. First, there could be accumulation of cocaine or a cocaine metabolite over time. Alternatively, the initial exposure to cocaine could stimulate new gene expression or change the activity of signaling components downstream of the amine receptors. We favor the latter model for several reasons. First, if sensitization were caused by accumulation, it is difficult to explain why sensitization would require a minimum time interval between doses, unless there were a slow metabolism to a more active form. We are not aware of any cocaine metabolites in vertebrates that are more active than cocaine itself. Second, we find that a similar minimum threshold dose is required both to generate moderate behavioral responses following a single cocaine exposure and also to show sensitization to a subsequent cocaine exposure. A requirement for new gene expression during the development of sensitization is consistent with studies in vertebrates, showing that cocaine exposure is associated with induction of several immediate-early genes, and additionally of genes encoding dynorphin, tyrosine hydroxylase and dopamine D1- and D2-like dopamine receptors [15–18].

The study of cocaine sensitization in rodent models is complicated due to effects of handling, familiarity of the testing environment, strain, stress from the procedures and anticipation of cocaine [19–21]. Furthermore, forward genetic approaches are difficult in these model animals. Simpler animals such as *Drosophila* may be less sensitive to these confounding influences. Many of the vertebrate monoamines, and their receptors (reviewed in [4]) and

transporters, have been isolated in *Drosophila*, including a cocaine-sensitive serotonin transporter [22,23]. Furthermore, cocaine-sensitive dopamine and octopamine transporter activities have been identified in the giant cockroach, *Blaberus giganteus* [24]. These observations suggest that the fundamental pathways involved in cocaine responses are conserved in invertebrates.

The behavioral responses to cocaine and the development of sensitization that we observe in *Drosophila* are strikingly similar to those seen in vertebrates, indicating conservation not only of the molecules involved in cocaine responses, but conservation in their linkage to reflexive behavioral output circuits. We propose the use of *Drosophila* as a new model system for the study of cocaine-induced motor behaviors and for the biological processes leading to sensitization. Forward genetic approaches feasible in this genetically tractable animal may elucidate the genes involved in both cocaine responsiveness and sensitization.

Materials and methods

Cocaine was volatilized from nichrome filaments, based on a design from Hatsukami's laboratory [25]: Ten turns of 28-gauge nichrome wire were formed by wrapping around a #8 nail, and crimp-connected to 12-gauge copper leads. The leads were passed through a #4 neoprene stopper, which fits tightly into a 25 × 95 mm fly vial. Individual filaments were calibrated by applying 1–5 volts d.c. from a low voltage/high current regulated power supply while a microprobe thermocouple was inserted into the filament. A solution (5 to 10 μl) of 10 mg/ml cocaine free-base in ethanol is applied to a nichrome filament and allowed to evaporate. After the ethanol had evaporated, the stopper with the imbedded filament was inserted into a 25 mm fly vial containing ~20 adult flies. The filament is heated to a voltage sufficient to reach 200°C within 5 sec. Sublimation of as little as 75 μg cocaine base is readily visible as a white smoke emanating from the filament. After 1 min contact with the volatilized cocaine, flies were transferred to a 3 × 3 × 0.7 cm glass viewing chamber for behavioral scoring. Images were recorded to videotape for subsequent behavioral scoring using a b/w 0.5 sec CCD camera (Hitachi KP-M1U) equipped with a 12.6–70 mm video zoom macro lens (Navitar) sufficient to capture the entire field of the viewing chamber. The flies were scored over a 5 min interval with the experimenter blind to the treatment group. All experiments were done during the light phase of flies entrained to a 12/12 light/dark cycle, using 2–5-day-old wild-type Oregon R flies.

Supplementary material

Quicktime videos of the behaviors described are published with this paper on the internet.

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