Dopamine modulates acute responses to cocaine, nicotine and ethanol in Drosophila
Roland J. Bainton*†, Linus T-Y. Tsai‡, Carol M. Singh§, Monica S. Moore§, Wendi S. Neckameyer§ and Ulrike Heberlein‡¥#

Background: Drugs of abuse have a common property in mammals, which is their ability to facilitate the release of the neurotransmitter and neuromodulator dopamine in specific brain regions involved in reward and motivation. This increase in synaptic dopamine levels is believed to act as a positive reinforcing and to mediate some of the acute responses to drugs. The mechanisms by which dopamine regulates acute drug responses and addiction remain unknown.

Results: We present evidence that dopamine plays a role in the responses of Drosophila to cocaine, nicotine or ethanol. We used a startle-induced negative geotaxis assay and a locomotor tracking system to measure the effect of psychostimulants on fly behavior. Using these assays, we show that acute responses to cocaine and nicotine are blunted by pharmacologically induced reductions in dopamine levels. Cocaine and nicotine showed a high degree of synergy in their effects, which is consistent with an action through convergent pathways. In addition, we found that dopamine is involved in the acute locomotor-activating effect, but not the sedating effect, of ethanol.

Conclusions: We show that in Drosophila, as in mammals, dopaminergic pathways play a role in modulating specific behavioral responses to cocaine, nicotine or ethanol. We therefore suggest that Drosophila can be used as a genetically tractable model system in which to study the mechanisms underlying behavioral responses to multiple drugs of abuse.

Background
Although psychostimulants, opiates and ethanol all have different primary effects and modes of action in the central nervous system (CNS), current theories suggest that their positive reinforcing, or rewarding, properties are mediated in part by an elevation of extracellular dopamine in the nucleus accumbens [1–4]. The associated acute locomotor-stimulating effects of these drugs have been proposed to model their rewarding qualities [5]. Selective destruction of dopaminergic neurons or pharmacological inhibition of dopaminergic systems prevents the stimulatory effects of most drugs of abuse; these manipulations also curtail drug self-administration [2,3]. For example, antagonists of dopamine D1 and D2 receptors block locomotor hyperactivity and self-administration of cocaine in rodents [6–11]. In addition, mice carrying a deletion of the dopamine D1 receptor or the dopamine transporter (DAT) are hyperactive and insensitive to the locomotor-activating effects of cocaine [12,13]. DAT mutant mice can still be trained to self-administer cocaine, however, and establish cocaine-conditioned place preference [14,15]. These results suggest that other neurotransmitter systems are also involved in the rewarding effects of cocaine.

Drosophila demonstrated dose-dependent behavioral responses. Low doses (50–75 µg) induced primarily grooming and hyperactivity. Moderate doses (100–150 µg) led to hypokinesis and stereotypic locomotion often manifested as circling. High doses (200–400 µg) induced spastic activity, tremor, and finally, complete loss of movement (akinesia). These behaviors are qualitatively similar to those described by McClung and Hirsh [16]. Our drug responses were approximately twofold weaker, however, that is, behaviors elicited by 200 µg cocaine in our laboratory are similar to those described for 100 µg by McClung and Hirsh [16]. The reasons for this discrepancy are not clear.
Although behaviors elicited were distinct at different cocaine doses, there was substantial individual variation among flies in a single experiment. In addition, individual flies often displayed a diversity of behaviors during any one-minute observation period, making unambiguous scoring and quantitative analyses difficult. To avoid these complications, we developed a simple climbing assay, based on the observation that cocaine exposure interferes with the fly’s normal propensity to negatively geotax in response to mechanical stimulation (see Materials and methods). Briefly, cocaine- or mock-exposed flies were introduced into a one-foot-long narrow cylinder and knocked to the bottom. Mock-treated flies responded by quickly climbing to the top of the cylinder. This response was reduced in a dose-dependent manner upon cocaine exposure. The proportion of flies that failed to climb, thus remaining at or near the bottom of the cylinder, was averaged over three consecutive trials, providing a quantitative and reproducible measure of the effect of the drug (Figure 1a). The (+) isomer of cocaine, which has reduced biologic activity in mammals [17], also showed reduced efficacy when tested in our climbing assay (Figure 1a). Flies recovered normal behavior within 15–20 minutes after exposure to moderate cocaine doses.

An overlapping, but not identical set of behaviors occurred when flies were exposed to volatilized nicotine. These behaviors included a very rapid onset of hyperactivity and spasmodic movements, leading to grooming, hypokinesia, and to akinesis in the most affected individuals. Repetitive locomotor behaviors were less obvious than in flies exposed to cocaine. Nonetheless, nicotine exposure impaired the flies’ ability to negatively geotax in a dose-dependent manner (Figure 1b). Flies recovered quickly, in approximately 5 minutes, after exposure to moderate nicotine doses (~6 µg). The actions of nicotine are likely to be CNS-specific because nicotinic acetylcholine receptors in insects are confined to the nervous system, where they appear to play a major excitatory role [18,19].

In summary, a simple startle-induced climbing assay allowed us to measure behaviors induced by cocaine or nicotine. The assay is quantitative, reproducible and shows specificity, as indicated by the reduced efficacy of (+) cocaine. It differs from direct observation of drug-exposed flies in that flies are subjected to mechanical stimulation in an environment conducive to negative geotaxis. Thus, the effect of the drug is measured by its ability to interfere with a normally robust fly behavior.

The role of dopamine in cocaine and nicotine responses

In mammals, cocaine inhibits the reuptake of monoaminergic neurotransmitters from the synaptic cleft through its interaction with plasma membrane monoamine transporters, including the transporters for dopamine (DAT), norepinephrine and serotonin [20]. Although the direct target of cocaine is not known in flies, a cocaine-sensitive serotonin transporter has been characterized [21,22]; a cocaine-sensitive dopamine transporter has not been identified to date.

To explore a role for dopamine in acute responses to cocaine and nicotine, we tested flies with severely reduced dopamine levels. This was achieved by feeding flies with 3IY, a dopamine-depleting compound [23]. Unlike mammals, Drosophila does not synthesize norepinephrine from dopamine [19]; therefore, 3IY treatment should specifically deplete dopamine. A complete loss of dopamine is incompatible with viability; for example, loss-of-function mutations in the Drosophila pale locus, which encodes TH [24], lead to embryonic lethality. In addition, prolonged 3IY treatment of larvae [23] and adults (our unpublished observations) causes akinesia and, eventually, death. Thus, our behavioral assays were carried out under conditions of partial dopamine loss, conditions that permitted normal baseline behaviors. For example, 3IY-treated flies displayed normal locomotion and geotaxis and performed normally in mock-exposure experiments [25] (see Materials and methods and below). But they showed a significant reduction, approximately 35%, in their sensitivity to the effects of cocaine and nicotine (Figure 2a). This effect was attributable to a decrease in dopamine levels, as L-Dopa treatment restored normal sensitivity to 3IY-treated flies (Figure 2a) under conditions that partially restored dopamine levels (Table 1).
L-Dopa treatment alone at this dose did not affect drug sensitivity (Figure 2a). Thus, a reduction in dopamine levels that did not affect basal locomotion or geotaxis resulted in diminished sensitivity to cocaine and nicotine, suggesting that dopamine mediates some of the acute effects of these drugs in *Drosophila*.

To obtain a more detailed view of the effect of dopamine depletion on cocaine-induced behaviors, we documented the locomotion patterns of control and 3IY-treated flies with a high-speed video analysis system. The flies were exposed to cocaine in the presence or absence of 3IY and L-Dopa. The locomotion patterns were assessed at various time points after exposure to cocaine.

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dopamine concentration (µg/ml)</th>
<th>Serotonin concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.76 ± 0.05 (10)</td>
<td>0.40 ± 0.08 (8)</td>
</tr>
<tr>
<td>3IY (10 mg/ml)</td>
<td>0.08 ± 0.01 (10)*</td>
<td>0.40 ± 0.08 (8)</td>
</tr>
<tr>
<td>3IY (10 mg/ml) + L-Dopa (1 mg/ml)</td>
<td>0.30 ± 0.04 (9)*</td>
<td>0.39 ± 0.08 (8)</td>
</tr>
<tr>
<td>L-Dopa (1 mg/ml)</td>
<td>1.46 ± 0.29 (10)*</td>
<td>0.37 ± 0.06 (8)</td>
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</table>

Table 1 shows treatment of *Drosophila* with 3IY or reserpine reduces dopamine but not serotonin levels. Twenty-five adult male flies were quick-frozen in liquid nitrogen and homogenized in 200 µl 0.1 M perchloric acid. Monoamine levels were measured using high pressure liquid chromatography (HPLC), as described by Neckameyer [23]. This procedure does not allow the quantification of local changes in monoamine levels. We suspect that the more dramatic reduction of dopamine in 3IY-treated flies is caused by the added depletion in the cuticle.

Although it is not possible to accurately quantify the overall effect of cocaine using the locomotor tracking system, we estimate that 3IY-treated flies exposed to 200 µg cocaine displayed behaviors that were similar to those induced by 100 µg cocaine in untreated flies. This approximately 50% reduction in the effects of cocaine is comparable to the 35% reduction measured in the climbing assay (Figure 2a).

In *Drosophila*, dopamine biosynthetic enzymes are expressed not only in the nervous system but also in the epidermis, where they participate in cuticle formation [26–28]. Therefore, HPLC measurements of dopamine levels in fly extracts (Table 1) reflect the combined levels in cuticle and nervous system.
and CNS. Although these data clearly established that 3IY and L-Dopa were ingested and had the predicted consequences, they did not allow us to quantify dopamine levels in the CNS. Unfortunately, dopamine levels in dissected brain preparations were too low for reliable measurement. With the purpose of reducing dopamine transmission specifically in the nervous system, we treated flies with reserpine. In mammals, reserpine efficiently depletes synaptic dopamine and other monoamines by inhibiting the vesicular monoamine transporter-2 (VMAT-2), thus preventing it from concentrating these neurotransmitters in synaptic vesicles [29]. Consistent with the notion that reserpine would not affect cuticle dopamine levels, flies treated with reserpine showed only a small but significant reduction in global dopamine levels, but surprisingly, no changes in global serotonin levels (Table 1). Like 3IY-treated flies, reserpine-treated flies showed a dose-dependent reduction in their response to cocaine (Figure 2b) while performing normally in mock-exposure experiments. A maximal reduction in sensitivity of 38% was achieved with 30 µM reserpine; higher doses reduced fly fitness, causing an apparent increase in cocaine sensitivity (data not shown). These data also suggest that Drosophila have a vesicular monoamine transporter that functions similarly to the mammalian transporter; sequences with strong homology to the mammalian reserpine-sensitive VMAT-2 gene have recently been identified in the Drosophila genome.

Cocaine-induced locomotor patterns of control and dopamine-depleted flies. Flies were exposed to 0, 100 or 200 µg cocaine, as indicated. Panels (a–f) and (g–l) correspond to vehicle-treated control (+ 3IY) and 3IY-treated (+ 3IY) flies, respectively. The drug effect increased gradually during the first 5 min after exposure; the locomotor patterns during the second (1–2) and fourth (3–4) min are shown to illustrate this effect. Treatment with 3IY had no obvious effect on mock-treated flies (0 µg cocaine). The effect of cocaine was reduced in 3IY-treated flies at all doses and times tested, however. See Table 2 for quantification of these data.
Why serotonin levels are not altered by reserpine treatment is unclear. In summary, we have shown that two mechanistically distinct pharmacological manipulations that reduce available dopamine render flies more resistant to the effects of cocaine. Whereas inhibition of dopamine biosynthesis with 3IY is known to alter dopamine levels in both the CNS and the cuticle [23], inhibition of the vesicular monoamine transporter by rexepine, and the resulting degradation of dopamine in synaptic terminals, should be CNS-specific. This suggests that the observed behavioral effects are caused by reduced levels of dopamine in the CNS rather than loss in the cuticle. Furthermore, we expect that compromised cuticle formation would, if anything, increase drug sensitivity, which is not the case. The monoamines tyramine and octopamine do not appear to be involved in regulating cocaine sensitivity, as flies containing mutations that interfere with the synthesis of these monoamines show normal cocaine responses ([30] and our unpublished observations). We cannot completely exclude a role for serotonin in regulating cocaine responsiveness. Although we found that serotonin levels appear normal in extracts from 3IY-treated flies (Table 1), it is possible that amine levels vary locally. Serotonin-specific immunohistochemical analysis of brains from 3IY-treated adult flies also failed to reveal detectable changes, however (data not shown).

Synergistic effects on behavior of cocaine and nicotine

Genetic or pharmacological manipulations that affect a common process often display dose-dependent synergistic interactions. Because our pharmacological depletion data suggested that both cocaine and nicotine exert their effects in part by modulating dopamine release, we tested for behavioral interactions between the two drugs. Simultaneous co-administration of low doses of cocaine and nicotine resulted in pronounced synergy in our behavioral assay (Figure 4). For example, only 1% and 2% of flies failed to climb when exposed to 1.5 µg nicotine or 50 µg cocaine, respectively (Figure 4a); a 59% climbing failure was achieved upon co-administration, an approximately 3.5-fold increase (Figure 4b). Thus, antagonism between the drugs was not observed.

Figure 4

Drosophila responds synergistically to co-administration of low doses of cocaine and nicotine. (a) The behavioral responses of wild-type flies to the indicated low doses of nicotine, cocaine, or both were measured in the negative geotaxis assay, as described in the Materials and methods (n = 9–15). (b) This synergistic response was significantly reduced (p < 0.001, n = 9) in flies in which dopamine levels had been reduced by 3IY treatment.

Table 2

<table>
<thead>
<tr>
<th>Cocaine (µg)</th>
<th>0</th>
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<th>200</th>
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<tr>
<td>Treatment</td>
<td>– 3IY</td>
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<td>Median velocity (mm/sec) 1–2 min 3IY</td>
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Treatment with 3IY reduces the sensitivity of flies to the locomotor-depressing and circling-inducing effects of cocaine. The median velocity (mm/sec) and median turning (°/mm) of flies exposed to various doses of cocaine is shown for the second (1–2 min) and fourth (3–4 min) minutes after exposure. Pairwise comparisons between 3IY-treated and vehicle-treated flies were carried out using the Wilcoxon–Mann–Whitney U test. Values in parentheses indicate the number of flies; asterisks denote statistically significant differences (p < 0.05). All pairwise comparisons between mock- and cocaine-treated flies showed highly significant differences (p ≤ 0.003).

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20-fold increase over an additive response. This synergy was greatly diminished in 3IY-treated flies (Figure 4b), which is consistent with the hypothesis that these drugs act in part through a common dopaminergic pathway. That response was not completely abrogated by 3IY is not surprising because this treatment did not fully deplete dopamine levels. In addition, these drugs might also synergize through non-dopaminergic pathways. These experiments do not establish whether cocaine and nicotine act on the same cells in the fly’s nervous system. If cocaine and nicotine acted completely independently, however, their combined administration would be expected to have additive effects.

In mammals, cocaine inhibits dopamine reuptake into dopaminergic terminals in the nucleus accumbens, whereas nicotine is believed to activate acetylcholine receptors, some of which are located presynaptically on dopaminergic neurons that project to the nucleus accumbens. Consistent with a convergent action of these drugs on nucleus accumbens dopamine regulation, cocaine and nicotine show an additive effect on dopamine release in the nucleus accumbens of freely moving rats [33]. In addition, self-administration of cocaine and nicotine has been shown to activate common regions in the mesocortico-limbic dopamine system in rats, as measured by induction of Fos-related protein expression [32]. Whether additive changes in dopamine levels in the nucleus accumbens lead to synergistic effects on relevant behavior has, to our knowledge, not been tested.

The role of dopamine in acute ethanol responses

The involvement of dopamine in acute drug responses can also be demonstrated with ethanol. When placed in a small chamber or in narrow tubes, flies displayed a basal level of locomotion (Figure 5ab, see Materials and methods). Upon exposure to ethanol vapor, they immediately increased their locomotor activity. After 7–10 minutes of hyperactivity, the flies became gradually less coordinated and increasingly sedated. After approximately 15 minutes, they stopped righting themselves. Flies treated with 3IY also showed an increase in locomotor activity upon ethanol exposure, but this effect was significantly reduced during the first 5 minutes of exposure (Figure 5ab). The effect of 3IY was reversed by simultaneously feeding L-Dopa. The duration of the locomotor-activating effects of ethanol and, consequently, the exposure time leading to sedation was not obviously changed by alterations in dopamine levels. Ethanol absorption was not modified by treatment with 3IY and/or L-Dopa (Figure 5c), arguing that dopamine depletion did not alter ethanol pharmacokinetics, but specifically reduced the flies’ response to ethanol-induced hyperactivity. Similarly, acute ethanol exposure increases dopamine release and locomotor activity in rodents [33,34]; the latter can be blocked by administration of dopamine receptor antagonists [35].

Conclusions

In summary, we have shown that upon acute exposure to cocaine, nicotine or ethanol, Drosophila displays a series of motor behaviors that are similar to those observed in...
mammals. In addition, we have presented evidence to show that, as in mammals, dopaminergic systems are involved in the manifestation of specific drug-induced behaviors. In contrast to our findings that dopamine-depleted flies showed decreased sensitivity to acute cocaine exposure, Li et al. [16] have observed increased cocaine sensitivity in flies in which evoked synaptic release of dopamine and serotonin neurons had been inhibited by targeted tetanus toxin expression. This apparent discrepancy could be explained by the fact that Li et al. used flies in which both dopamine and serotonin neurons were affected throughout development, while our short-term pharmacological manipulations of fully developed adult flies did not seem to target serotonin neurons. Recent studies using dopamine receptor antagonists in a decapitated preparation of Drosophila have implicated dopaminergic systems in locomotion and grooming [37] and the responses to cocaine and cocyclohydrine [38]. Taken together, these studies show not only that behaviors induced by acute drug exposure are surprisingly similar in flies and mammals, but also that at least some of the neurotransmitter systems that mediate these behaviors are conserved. We therefore suggest that Drosophila can be used as a genetically tractable model system in which to study the mechanisms underlying behavioral responses to multiple drugs of abuse.

Materials and methods

Drosophila culture and behavioral assays

Adult male Drosophila melanogaster of the Canton-S wild-type strain were used for all experiments. These flies were grown on cornmeal-molasses food at 25°C and 70% relative humidity. Cocaine (free base, Sigma), (+) cocaine (NDA) and nicotine (Sigma) solutions were prepared by dissolution in ethanol (cocaine) or water (nicotine). Drugs were delivered as described by McGeer and Hish [16]. Flies were exposed for 1 min, then placed into glass column (23.5 cm long, 2.5 cm diametere) lined with nylon mesh (250 microns, Monofilament Cloth). Columns were knocked on a soft surface to force flies to the bottom. After 1 min, flies remaining within 1 cm of the bottom were counted; this procedure was performed three times at 1 min intervals. Mock-treated flies responded by rapidly climbing back to the top of the column, presumably as a response to starvation and because of their normal motivation to negatievolently. Drug-treated flies showed reduced climbing as they performed the various abnormal locomotor behaviors described in the test. Drug-treated flies showed little or no recovery during the 3 min of the assay. The effect observed is the average (over 3 min) of the number of flies that remained near the bottom and is expressed as a percentage of the total number of flies. These scores distribute normally with respect of the mean and were analyzed statistically using Student’s paired t-test.

Drug feeding protocols

Pharmacological treatment with 3IY (Sigma) and L-Dopa (Sigma) was carried out as described by Neckameyer [23]. L-Dopa and 3IY were dissolved in aqueous 5% sucrose, 2% yeast solution and a saturating amount (1.2 ml) was added to glass vials lined with 5 MM paper. Newly eclosed males were kept in these vials for 40–48 h at 25°C and 90% relative humidity before drug exposure. Reserpine (Sigma) was prepared as above and flies were treated for 24 h. Upon mock exposure, flies treated with 3IY and/or L-Dopa displayed behaviors indistinguishable from vehicle-treated flies.

Ethanol-induced behaviors

Adult male flies were exposed to 3IY and/or L-Dopa for 48 h as described above. Flies were introduced, four at a time, into a 6 × 8 × 1.5 cm acrylic exposure chamber and allowed to rest for 1 min. They were then videotaped during exposure to a constant flow of air for 5 min followed by ethanol vapor (approximately 15 mM) for 25 min, as described previously [39]. Exposure chambers were covered with a grid of orthogonal lines spaced 1.5 cm apart. The locomotor activity of each individual fly was quantified manually as the number of lines crossed in 1 min intervals. Basal locomotion without ethanol was measured in narrow perforated plastic tubes (3-mm diameter, 6 cm length) and expressed as the number of lines (at 1 cm intervals) crossed per minute. The flies’ wings were clipped 24 h before testing to avoid flight during the ethanol exposure.

Locomotor tracking system

Five adult male flies were exposed to volatilized cocaine for 15 sec and placed in a 15 cm × 4 cm observation chamber. They were videotaped at a capture rate of 15 frames/sec beginning 30 sec after exposure. Traces were generated using the Dynamic Image Analysis System (Softisc Inc.). Paths joining contiguous traces were graphically represented over one minute intervals as dots (center of trace) connected by lines (path traveled between frames). From these paths, the velocity of movement (mm/sec) and the degree of turning or circling (T mm) were calculated and subjected to pairwise statistical comparisons using the Wilcoxon-Mann-Whitney test (velocity and turning of cocaine-exposed flies do not distribute normally). Flies that failed to move during the 1 min period were eliminated from the calculations of turning.

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References


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