

RESEARCH ARTICLE

Morphine addiction in ants: a new model for self-administration and neurochemical analysis

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ABSTRACT

Conventional definitions of drug addiction are focused on characterizing the neurophysiological and behavioral responses of mammals. Although mammalian models have been invaluable in studying specific and complex aspects of addiction, invertebrate systems have proven advantageous in investigating how drugs of abuse corrupt the most basic motivational and neurochemical systems. It has recently been shown that invertebrates and mammals have remarkable similarities in their behavioral and neurochemical responses to drugs of abuse. However, until now only mammals have demonstrated drug seeking and self-administration without the concurrent presence of a natural reward, e.g. sucrose. Using a sucrose-fading paradigm, followed by a two-dish choice test, we establish ants as an invertebrate model of opioid addiction. The ant species *Camponotus floridanus* actively seeks and self-administers morphine even in the absence of caloric value or additional natural reward. Using HPLC equipped with electrochemical detection, the neurochemicals serotonin, octopamine and dopamine were identified and subsequently quantified, establishing the concurrent neurochemical response to the opioid morphine within the invertebrate brain. With this study, we demonstrate dopamine to be governing opioid addiction in the brains of ants. Thus, this study establishes ants as the first non-mammalian model of self-administration that is truly analogous to mammals.

KEY WORDS: Dopamine, Serotonin, Octopamine, High pressure liquid chromatography, Electrochemical detection, Drug seeking, Sucrose-fading procedure, Two-dish choice test, Invertebrate

INTRODUCTION

Drug abuse presents a fundamental paradox in human behavior. When individuals become addicted, they consume drugs of abuse despite harmful consequences. Conventional studies of drug abuse and addiction are primarily focused on characterizing human neuronal and behavioral responses. Historically, mammalian models have provided insights into the modulation of natural behavior throughout the addiction process by studying specific behaviors associated with addiction, such as drug seeking and self-administration (Kaun et al., 2012). However, invertebrate systems have recently proven advantageous to investigate basic motivational and behavioral systems underlying addiction (Søvik

et al., 2013; Barron et al., 2009; Søvik and Barron, 2013; Nathaniel et al., 2009).

Invertebrates and mammals have been shown to exhibit remarkable similarities between behavioral and neurochemical responses to drugs of abuse (Barron et al., 2009, 2010). Cocaine can modulate bees' responses to natural floral rewards (Barron et al., 2009), while amphetamine, cocaine and morphine modulate locomotor activity in crayfish (Nathaniel et al., 2009; Huber et al., 2011). The presence of drug-seeking behavior in crayfish, honeybees and *Drosophila* also indicates that addiction is evolutionarily ancient (Barron et al., 2009; Nathaniel et al., 2010).

In *Drosophila*, addiction to and self-administration of ethanol have been shown; however, this requires the drug to be coupled with a caloric/natural reward, i.e. sucrose (Kaun et al., 2012; Ja et al., 2007; Devineni and Heberlein, 2009; Xu et al., 2012). Furthermore, *Drosophila* naturally seek ethanol, a main metabolite of fermenting fruit, which also acts as a long-distance signal for *Drosophila* mating sites (Kaun et al., 2012). In addition, as ethanol has caloric value, addiction and self-administration are further confounded with natural foraging (Devineni and Heberlein, 2009).

Consequently, this study establishes ants as the first invertebrate model to exhibit self-administration in the absence of an additional caloric reward. We hypothesized that the unique foraging and navigation abilities of ants can be co-opted by addictive pathways to elicit self-administration and utilized to assess the rewarding properties of a drug in the absence of natural rewards.

Individual ant foragers are known to memorize the location of a feeding site, and reliably visit the location for repeated rewarding visits (Wolf, 2008; Wolf and Wehner, 2000). Additionally, ants are social, complex organisms that rely on interactions within a colony to facilitate foraging bouts (Holdobler and Wilson, 1990). These bouts are reinforced by complex behavioral repertoires (Seid and Traniello, 2006) and advanced learning abilities (Holdobler and Wilson, 1990). Ants also have well-characterized neural systems (Gronenberg et al., 1996; Gronenberg, 2001; Hoyer et al., 2005; Seid and Traniello, 2005; Seid et al., 2008; Seid and Wehner, 2008, 2009; Muscedere et al., 2012), allowing us to pinpoint the specific neuronal circuits associated with addictive behavior: specifically, the expression and synaptic activity of the neurotransmitters dopamine (DA), octopamine (OA) and serotonin (5-HT), which are tractable in invertebrate models, such as ants (Seid and Traniello, 2005; Seid et al., 2008; Seid and Wehner, 2008, 2009; Muscedere et al., 2012). These pathways play not only important roles in the maintenance of an organism's response to rewarding stimuli but also a crucial role in the maintenance of other natural behaviors (Seid and Traniello, 2005; Seid et al., 2008; Seid and Wehner, 2008, 2009; Muscedere et al., 2012).

Using high pressure liquid chromatography (HPLC) equipped with electrochemical detection (ED), we quantified 5-HT, DA and OA titers from whole ant brains. Quantifying the biogenic amines verified that the observed preference for morphine in morphine-

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trained ants is due to its addictive pharmacological effects. By simultaneously investigating self-administration and quantifying the expression of the biogenic amines, we establish ants as the first linked behavior/neurochemical model of invertebrate opioid addiction.

MATERIALS AND METHODS

Study species and animal care

Three established queen-right colonies (approximately 500 individuals including eggs and larvae) of the ant species *Camponotus floridanus* (Buckley), collected in Gainesville, FL, USA, in March 2015, were used as our source of ants. These three colonies were reared in uniform conditions of 25°C and a 12 h:12 h light:dark cycle, and received food (live insects and sugar water) *ad libitum*.

Housings and feeders

The experimental housings were fabricated out of 20.32 cm round plastic Petri dishes (Fig. 1). Each environment contained a centrally located glass test tube (10×75 mm) that, based on previous work, ants readily adapt as their home chamber. Throughout the experiment, ants were allowed to naturally forage to a feeder (cap from a 1.5 ml microcentrifuge tube) located at one of two set positions, A or B (Fig. 1). The two feeding locations were equidistant from the entrance of the home chamber, either 10 cm to the left or right. The feeding site was randomized daily using a research randomizer (www.randomizer.org). Feeders contained 100 µl of solution and were placed into and removed from the environment daily.

Video recordings and scoring

Video recordings for analysis of ant behavior were captured using a Sony HD Handycam (HDR-PJ430V), and a Logitech HD Web Cam (960-000683 HD Pro Webcam B910) connected to an Apple Mac mini (MC438xx/A). Both cameras were mounted above the testing site and recorded 4 h behavioral observations during a controlled feeding time.

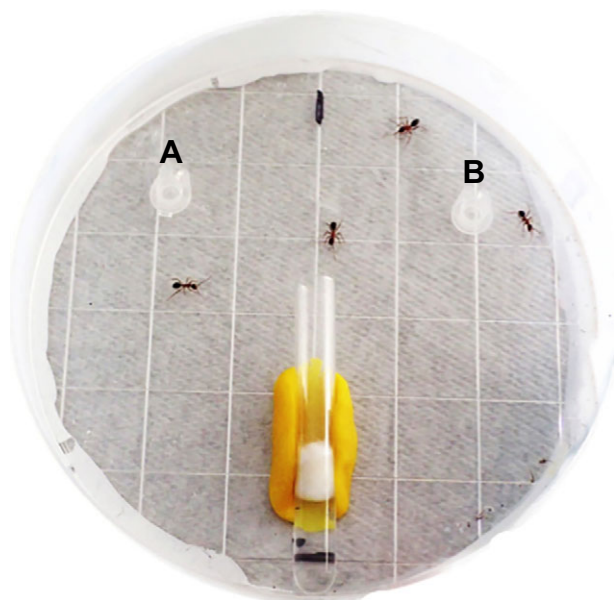


Fig. 1. Experimental housing and feeders. Ants were housed in 20.32 cm round plastic Petri dishes containing a centrally located glass test tube (10×75 mm) and two feeders (A and B). Solutions were randomized between the two feeding locations.

Recorded footage was analyzed manually. The total number of visits to a feeder was scored for 60 min following the first foraging event. A foraging event was only counted once an ant climbed into the feeder and extended her proboscis. In order for repeated visits to be counted, the individual had to either return to the nest tube or recruit a nest mate.

Experimental design

Prior to the start of the experiment, 90 experienced foragers were randomly selected from a feeding dish (30 from each of the three breeding colonies). The ants were then divided into 18 groups of five ants each and placed in experimental housings. Each group of five ants was designated as one behavioral unit ($N=6$ per condition). Once separated into their test groups, ants acclimated to their new environment for 24 h, during which time food was withdrawn (starvation period).

The experiment consisted of three conditions: (1) sucrose-fading procedure, (2) morphine exposure and (3) drug naive control. The sucrose-fading and morphine-exposure experiments ran for 7 days; these experiments both incorporated a sucrose-fading procedure during days 1–6.

Sucrose-fading procedure

During our sucrose-fading paradigm, the sucrose concentration in solution was decreased twice (Table 1). On the first 2 days, 1.0 mol l⁻¹ sucrose (aq) was administered. On days 3 and 4, 0.5 mol l⁻¹ sucrose (aq) was administered. Lastly, on days 5 and 6, pure 100% H₂O, i.e. no sucrose, was administered. Therefore, all caloric value was removed from the solution.

Ants in the sucrose-fading (control) groups (sucrose-trained ants) experienced a sucrose-fading paradigm that directly mirrored the reduction of sucrose in the morphine-exposed groups (Table 1; see below). This experiment was a control to determine whether the reduction in sucrose caused any change in behavior.

Morphine exposure

Morphine-exposed groups were subjected to increasing morphine concentrations while simultaneously experiencing a reduction in sucrose (Table 1), as described above. Morphine dosages administered in this experiment were determined using a dose-response curve for oral morphine administration, in children, and scaled down to the average mass of the ant (Wong et al., 2012).

During morphine exposure, ants were dosed with 0.06 mg ml⁻¹ morphine in 1.0 mol l⁻¹ sucrose (aq) for the first 2 days. Ants were then dosed with 0.12 mg ml⁻¹ morphine in 0.5 mol l⁻¹ sucrose (aq) for the next 2 days. On the last 2 days, ants were administered 0.12 mg ml⁻¹ morphine (aq) without sucrose, in 100% H₂O.

Drug naive control

The drug-naive groups were controls established to ensure ants did not exhibit an innate preference for morphine. Therefore, drug-naive

Table 1. Sucrose-fading procedure (days 1–6) for sucrose-fading and morphine-exposed ants

	Days 1 and 2	Days 3 and 4	Days 5 and 6
Sucrose fading	1.0 mol l ⁻¹ sucrose (aq)	0.5 mol l ⁻¹ sucrose (aq)	H ₂ O (no sucrose)
Morphine exposed	0.06 mg ml ⁻¹ morphine in 1.0 mol l ⁻¹ sucrose (aq)	0.12 mg ml ⁻¹ morphine in 0.5 mol l ⁻¹ sucrose (aq)	0.12 mg ml ⁻¹ morphine in H ₂ O (no sucrose)

groups did not experience the 6 day sucrose-fading procedure. Instead, drug-naive ants only participated in the two-dish choice test.

Two-dish choice test

A well-established sucrose-fading procedure was modified, followed by a two-dish choice test to initiate self-administration in the absence of a caloric reward (Roitman et al., 2004). A two-dish choice test (or two-dish design) presents the organism with a choice between two randomized feeders containing either a drug (morphine) or natural reward (sucrose).

After the sucrose-fading procedure, ants were tested for their preference for 0.5 mol l⁻¹ sucrose (aq) versus a pure H₂O solution (0.0 mol l⁻¹ sucrose), the natural reward and control solution, respectively. Meanwhile, both morphine-exposed and drug-naive ants were tested for their preference for 0.5 mol l⁻¹ sucrose (aq) versus a pure 0.12 mg ml⁻¹ morphine (aq) solution, the natural reward and drug reward, respectively.

Brain preparations and HPLC-ED neurochemical analysis

Following the two-dish choice test, whole brains were dissected in PBS and stored in 80 µl of mobile phase at -80°C to prevent amine degradation. The mobile phase consisted of 50 mmol l⁻¹ citric acid buffered with 2.5 g sodium acetate at pH 3.6, 24% acetonitrile, 750 ml nanopure H₂O, 100 µl of triethylamine and 0.43 g of sodium dodecyl sulfate (SDS) (Hardie and Hirsh, 2006; Smith et al., 2013; Muscedere et al., 2013). Prior to quantification, individual brains were homogenized in the mobile phase and centrifuged for 10 min at 10,000 rpm and -9°C (Hardie and Hirsh, 2006; Smith et al., 2013; Muscedere et al., 2013).

The HPLC-ED system consisted of a LC-20AD pump, a Waters Symmetry C-8 5 µm column (part no. WAT054270), and a CoulArray Electrochemical Detector (ESA, Inc.). The second and third channels of the detector were set to 450 and 650 mV, respectively. A 20 µl sample of supernatant liquid from each brain sample was injected onto the HPLC column and amine levels were detected on both the 450 and 650 mV channels (Hardie and Hirsh, 2006; Smith et al., 2013; Muscedere et al., 2013); 500 nmol l⁻¹ and 1, 5 and 10 µmol l⁻¹ mixtures of OA, DA and 5-HT standards in mobile phase (aq) were run at both the beginning and end of each day in order to properly quantify amines via the production of standard curves.

Statistical analysis

We used SPSS V.21 and R-Studio for our general linear mixed model (GLMM), univariate general linear model (GLM), *t*-test and *post hoc* Tukey HSD. Alpha was set at 0.05. A 'ratio of visits' for the two-dish choice test was calculated during our analysis to equalize the data. The equation to derive the ratio was as follows: ratio of visits=[(average number of visits to an individual feeder)÷(average total visits between the two feeders)].

RESULTS

Sucrose-fading procedure (days 1–6)

During the first 2 days of the sucrose-fading procedure, we saw a sharp decrease in visits to feeders by both sucrose-trained and morphine-trained ants due to the 24 h starvation period prior to the experiment (Fig. 2). On day 3, there was a slight increase in the number of visits to feeders by the morphine-trained ants due to the first reduction in sucrose and the concurrent increase in morphine concentration (Fig. 2, Table 1); however, an overall downward trend in visitations continued on day 4. On day 5, a divergence between the two treatment groups emerged in which

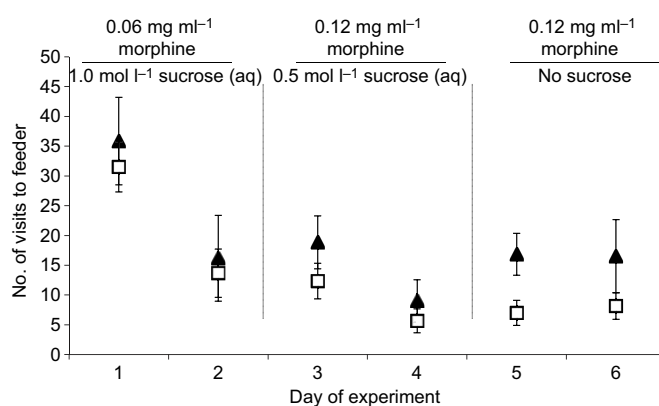


Fig. 2. Comparison of feeding behavior during the 6 day sucrose-fading procedure. Data (means±s.e.m.) are shown for sucrose-trained (squares) and morphine-trained (triangles) ants (see Table 1). *t*-test: $T=-2.4019$, d.f.=10, $P=0.0372$.

morphine-trained ants significantly increased visitation rates to their feeder compared with sucrose-trained ants (Tables S1, S2; Fig. 2; $T=-2.4019$, d.f.=10, $P=0.0372$). This trend continued on day 6, although the results were not significant because of variance within the morphine-trained groups (Tables S1, S2; Fig. 2; $T=-1.2736$, d.f.=10, $P=0.2316$).

Two-dish choice test

Prior to analysis, a GLMM showed there was no colony effect ($Z=0.659$, $P=0.510$) and a main effect of morphine treatment ($Z=2.550$, $P=0.011$). We concluded that we could reject the possibility of colony as a random effect and ran a univariate GLM. Remarkably, the morphine-trained ants exhibited a similarity to mammals in terms of their behavioral response to the drug of abuse (Carrillo et al., 2008), with 65% of the ants foraging to the morphine feeder over a natural reward, sucrose (Table S3; Fig. 3; $F_{1,17}=3.464$, $P=0.041$). Drug-naive ants, however, did not exhibit a preference for morphine and were not statistically distinguishable from sucrose-trained ants (Tables S4, S5; Fig. 3; Tukey's HSD $P=0.739$). Furthermore, morphine-trained ants sought pure morphine and were significantly different from both sucrose-trained and naive ants, which expressed a strong preference for sucrose (Fig. 3; Tukey's HSD $P=0.016$ and $P=0.005$, respectively).

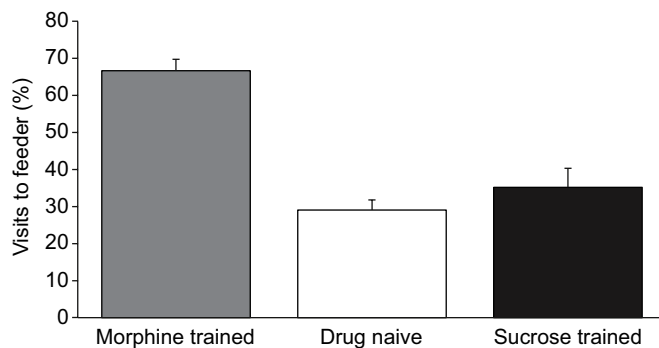


Fig. 3. Two-dish choice test. Data (means±s.e.m.) show the percentage of visits to a feeder containing either 0.12 mg ml⁻¹ morphine (morphine trained and drug naive) or H₂O (sucrose trained) versus 0.5 mol l⁻¹ sucrose (aq). Morphine-trained ants visited morphine feeders at twice the rate of drug-naive and sucrose-trained ants. $N=6$ per condition, $N=18$ total. Univariate general linear model; $F_{1,17}=3.464$, $P=0.041$.

HPLC-ED neurochemical analysis

Prior to analysis, a univariate GLM showed there was no colony effect ($F_{2,2}=0.9103$, $P=0.4126$) and only a main effect of treatment. It was determined that morphine-trained ants exhibited a significant increase in DA compared with both sucrose-trained and drug-naive ants (Fig. 4A; $F_{2,32}=14.42$, $P\leq 0.001$). Drug-naive ants, however, were indistinguishable from sucrose-trained ants (Fig. 4A; Tukey's HSD $P=0.363$). There was no significant difference between morphine-trained, sucrose-trained or drug-naive ants in regards to either OA or 5-HT titer (Fig. 4B;

$F_{2,35}=0.245$, $P=0.784$; and Fig. 4C; $F_{2,25}=0.510$, $P=0.607$, respectively).

DISCUSSION

Here, we established ants as a viable model of invertebrate opioid addiction. Our ants actively sought and self-administered morphine even in the absence of caloric value and during an unlimited access two-dish choice test. We found that the biogenic amine DA significantly increased in morphine-trained ants, a result similar to findings in mammals. Thus, this study establishes ants as the first non-mammalian model of opioid self-administration and addiction that is truly analogous to mammals.

It is well established that the phenomenon of seeking is expressed in an increased number of visits to feeders containing drugs of abuse (Huber et al., 2011; Nathaniel et al., 2010; Carrillo et al., 2008; Wise, 1997). This was first seen within our morphine-exposed ants during the sucrose-fading procedure. On days 5 and 6, once all caloric value had been removed, morphine-trained ants significantly increased, and maintained, visitation rates to their feeder. When compared with days 3 and 4, i.e. when sucrose was still present in the morphine solution (Table 1), the observed increase in the number of visits demonstrates that morphine-exposed ants were seeking morphine (Fig. 2). The large variance in visitations observed on day 6 is expected as some ants will require more or less morphine than others because of ingesting this concentration for 4 days, i.e. tolerance.

We found a complete reversal of natural behavior, in which morphine-trained ants sought pure morphine, whereas both sucrose-trained and naive ants maintained a strong preference for sucrose (Fig. 3). Rat models established that a sucrose-fading procedure followed by an unlimited access two-dish design was an effective method to demonstrate self-administration (Roitman et al., 2004) and we now establish the first invertebrate model. Preference for a drug solution without a natural reward is the fundamental crux demonstrating that ants are addicted and self-administer. Furthermore, we were able to override the ants' physiological preference for sucrose and replace it with a preference for morphine in our study, even in the presence of unlimited sugar.

Recently, it has been shown that drugs of abuse influence the brains of invertebrates by modulating the same neurotransmitters as in mammals (Barron et al., 2010); specifically, the neurotransmitters DA, 5-HT and OA (Kaun et al., 2012; Barron et al., 2010; Søvik and Barron, 2013). Although DA is known to be responsible for reward across a plethora of animal models, its role in signaling the invertebrate response is less clear. Similar to its function in mammals, DA may be responsible for seeking and reward behavior in ants (Gronenberg et al., 1996; Seid et al., 2008; Seid and Wehner, 2008, 2009; Muscedere et al., 2012; Hardie and Hirsh, 2006). In contrast, in more closely related invertebrates, OA and DA modulate different behavioral systems, with OA being responsible for rewarding stimuli and DA governing aversive reward (Kaun et al., 2012; Barron et al., 2009, 2010; Søvik and Barron, 2013).

Remarkably, we found that DA titer significantly increased in the brains of morphine-treated ants, a result not seen in either sucrose-trained or drug-naive ants (Fig. 4A). When paired with the behavioral data, the observed increase of DA titer in morphine-trained ants supports the hypothesis that DA is largely governing opioid reward in ants. During our two-dish choice test, an increase was seen in the number of visits to the morphine feeder (Fig. 3) and we now establish a concurrent increase in DA (Fig. 4A). An analogous response has been observed in mammals, where the

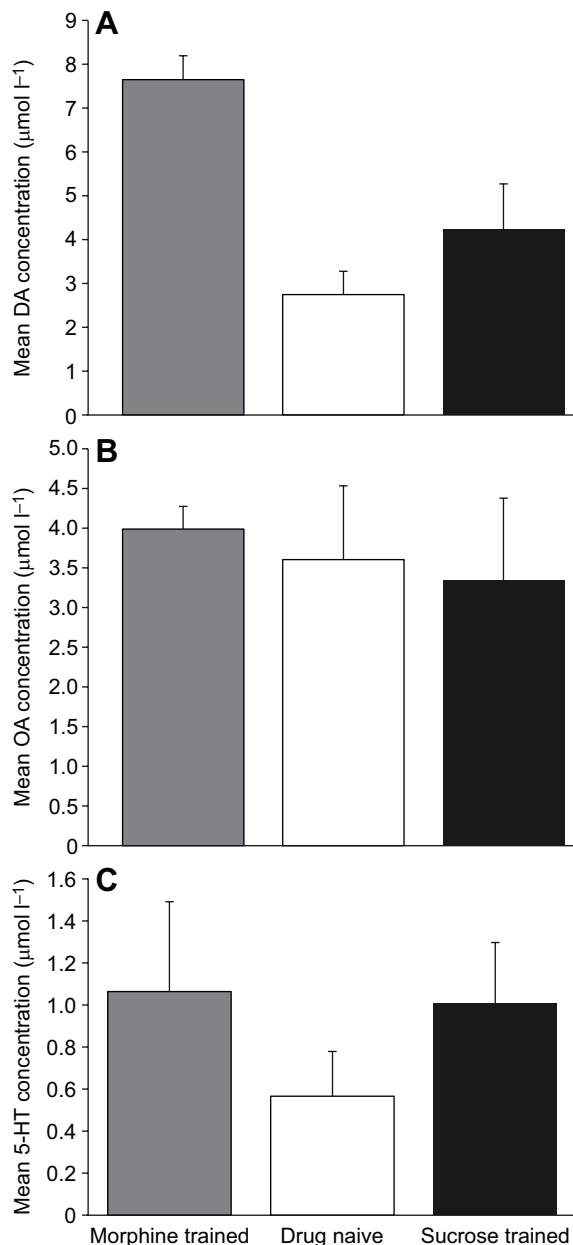


Fig. 4. Neurochemical analysis. (A) Mean (\pm s.e.m.) dopamine (DA) concentration for morphine-trained ($N=15$), drug-naive ($N=14$) and sucrose-trained ($N=12$) ants. Univariate general liner model; $F_{2,32}=14.42$, $P\leq 0.001$. (B) Mean (\pm s.e.m.) octopamine (OA) concentration for morphine-trained ($N=13$), drug-naive ($N=14$) and sucrose-trained ($N=10$) ants. Univariate general liner model; $F_{2,35}=0.245$, $P=0.784$. (C) Mean (\pm s.e.m.) serotonin concentration for morphine-trained ($N=9$), drug-naive ($N=10$) and sucrose-trained ants ($N=6$). Univariate general liner model; $F_{2,25}=0.510$, $P=0.607$.

release of DA into the mammalian brain directly increases reward-seeking behavior (Roitman et al., 2004; Wise, 1997).

Our findings, however, seem to be in contrast to the accepted role of OA and 5-HT in invertebrate reward (Søvik and Barron, 2013; Barron et al., 2010; Huber et al., 2011) as neither was influenced by opioid treatment (Fig. 4B,C). However, all previous studies investigating invertebrate reward circuitry have utilized sucrose as part of the rewarding stimuli to elicit a robust behavioral and neurochemical response (Søvik and Barron, 2013; Barron et al., 2010; Huber et al., 2011), not pure morphine. As all of our experimental conditions included exposure to sucrose, we cannot determine whether sucrose exposure influenced baseline DA, OA or 5-HT titer. Future experiments may determine whether sucrose alone, as a natural reward, elicits a significant influence on baseline biogenic amine concentration. Such a study, however, would not influence our current results and we do not believe OA or 5-HT plays an integral role in invertebrate opioid addiction.

Acknowledgements

We would like to thank The University of Scranton, Department of Biology, for funding this research. We wish to acknowledge Tim Corcoran for helping us score the behavioral tests. It is with the utmost gratitude that we thank Dr David Rusak and Dr Joe Vinson from The University of Scranton's Department of Chemistry for supporting this research and aiding in the HPLC-ED procedure. We also extend our deepest appreciation to Dr Patrick Orr from The University of Scranton's Department of Psychology for helping with the statistical analysis.

Competing interests

The authors declare no competing or financial interests.

Author contributions

B.V.E. and M.A.S. were responsible for experimental design, methodology, analysis and manuscript preparation. J.T.C. was responsible for experimental design, analysis and drug oversight. B.V.E. ran all experiments with the help of undergraduate researchers.

Funding

This research was funded via internal grants from The University of Scranton.

Data availability

Neurochemical data are available upon request via email.

Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.140616.supplemental>

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Table S1: Raw Data: Morphine-Exposed (Days 1 to 6)

	0.06 mg/mL Morphine in 1.0 M (aq) Sucrose		0.12 mg/mL Morphine in 0.5 M (aq) Sucrose		0.12 mg/mL Morphine in H₂O (aq)	
Colony 1 - A	27	11	4	13	23	40
Colony 1 - B	38	11	12	2	9	3
Colony 2 - A	25	1	13	3	4	0
Colony 2 - B	35	5	26	6	23	11
Colony 3 - A	70	50	33	25	17	19
Colony 3 - B	20	19	25	5	25	26

Table S2: Raw Data: Sucrose-Fading (Days 1 to 6)

	1.0 M (aq) Sucrose		0.5 M (aq) Sucrose		H₂O (aq)	
Colony 1 - A	30	29	26	6	5	7
Colony 1 - B	44	15	13	3	12	4
Colony 2 - A	30	6	4	2	0	0
Colony 2 - B	43	16	10	2	9	13
Colony 3 - A	18	0	10	15	13	14
Colony 3 - B	24	16	11	6	3	11

Table S3: Raw Data: Morphine-Exposed (Two-dish choice test)

	0.5 M (aq) Sucrose	0.12 mg/mL Morphine in H₂O (aq)
Colony 1 - A	8	24
Colony 1 - B	5	5
Colony 2 - A	1	5
Colony 2 - B	10	19
Colony 3 - A	9	4
Colony 3 - B	16	33

Table S4: Raw Data: Drug-Naive (Two-dish choice test)

	0.5 M (aq) Sucrose	0.12 mg/mL Morphine in H₂O (aq)
Colony 1 - A	21	7
Colony 1 - B	14	9
Colony 2 - A	92	46
Colony 2 - B	26	8
Colony 3 - A	65	21
Colony 3 - B	21	5

Table S5: Raw Data: Sucrose-Fading (Two-dish choice test)

	0.5 M (aq) Sucrose	H₂O (aq)
Colony 1 - A	4	1
Colony 1 - B	12	7
Colony 2 - A	5	6
Colony 2 - B	14	9
Colony 3 - A	28	15
Colony 3 - B	27	5