



# D-amphetamine stimulates unconditioned exploration/approach behaviors in crayfish: Towards a conserved evolutionary function of ancestral drug reward<sup>☆</sup>

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## ABSTRACT

In mammals, rewarding properties of drugs depend on their capacity to activate a dopamine-mediated appetitive motivational seeking state—a system that allows animals to pursue and find all kinds of objects and events needed for survival. With such states strongly conserved in evolution, invertebrates have recently been developed into a powerful model in addiction research, where a shared ancestral brain system for the acquisition of reward can mediate drug addiction in many species. A conditioned place preference paradigm has illustrated that crayfish seek out environments that had previously been paired with psychostimulant and opioid administration. The present work demonstrates that the administration of D-amphetamine stimulates active explorative behaviors in crayfish through the action of the drug within their head ganglion. Crayfish, with a modularly organized and experimentally accessible, ganglionic nervous system offers a unique model to investigate (1) the fundamental, biological mechanisms of addictive drug reward; (2) how an appetitive/seeking disposition is implemented in a simple neural system, and (3) how it mediates the rewarding actions of major drugs of abuse.

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## 1. Introduction

Invertebrates have emerged as useful animal models of addiction (see review in Wolf and Heberlein, 2003). With comparatively simple nervous systems and amenability to genetic manipulations, such models have advanced studies of the molecular underpinnings of behavioral responses to drugs, including acute responses, tolerance, withdrawal and sensitization (Morgan and Sedensky, 1995; McClung and Hirsh, 1999; Singh and Heberlein, 2000; Schafer, 2004; Scholz, 2005; Nichols, 2006; Morozova et al., 2006; Raffa et al., 2006; Feng et al., 2006; Huber et al., 2011).

Many invertebrates are susceptible to developing drug-seeking behaviors as they show a preference for a variety of human drugs of abuse. Rewarding properties have been demonstrated across different invertebrate taxa for psychostimulants (Wolf, 1999; Kusayama and Watanabe, 2000; Panksepp and Huber, 2004), opioids (Srivastava and Singh, 2006; Nathaniel et al., 2009a, 2009b), alcohol (Parsons, 1979; Bellen, 1998; Cadieu et al., 1999; Abramson et al., 2000, 2004),

nicotine (Singaravelan et al., 2005), and caffeine (Singaravelan et al., 2005). Drugs of abuse also promote unconditioned behavioral responses similar to those in mammals, including stereotypical movements, increased locomotor activity, and consummatory behaviors (Wong et al., 1991; Morgan and Sedensky, 1995; McClung and Hirsh, 1998; Singh and Heberlein, 2000; Bainton et al., 2000; Rothenfluh and Heberlein, 2002; Dimitrijevic et al., 2004; Raffa and Martley, 2005; Feng et al., 2006). Many of these responses, following repeated drug administrations, are susceptible to sensitization (McClung and Hirsh, 1998; Wolf, 1999; Wolf and Heberlein, 2003; Hou et al., 2004; Dimitrijevic et al., 2004; Scholz, 2005).

Among the wide range of drug-elicited behavior, exploration and approach signify an appetitive motivational state evident when animals seek natural rewards such as food, water, sexual stimuli, and secure environments (Glickman and Schiff, 1967; Trowill et al., 1969; Panksepp, 1981, 1998; Ikemoto and Panksepp, 1999; Alcaro et al., 2007). In mammals, it has been suggested that the rewarding properties of drugs arise out of an abnormal stimulation of the neural processes involved in the activation of appetitive dispositions—the SEEKING system (Wise and Bozarth, 1987; Robinson and Berridge, 1993; Panksepp et al., 2002). Invertebrates, which exhibit similar dispositions during naturalistic contexts, represent useful models for identifying and manipulating the neural circuits responsible for exploration and investigation (from sensory input to motor output), and to characterize how drugs of abuse may affect such activities. The neural circuitry of forward locomotion in the brain of *Caenorhabditis*

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*elegans* has recently been identified, along with some of the external stimuli that influence movements toward favorable conditions of chemotaxis, thermotaxis, and aerotaxis (Gray et al., 2005). Dopamine and glutamate serve as key modulators of such circuits (Hills et al., 2004), and it is possible that some of the rewarding properties of drugs derive from their capacity to stimulate their relevant, underlying networks (Panksepp, 1998; Ikemoto and Panksepp, 1999; Alcaro et al., 2007).

The present paper aimed to characterize the effects of amphetamine on invertebrate appetitive/exploratory behaviors. In a conditioned place preference (CPP) paradigm, crayfish preferred environments that had been associated with cocaine or amphetamine (Panksepp and Huber, 2004; Huber, 2005). However, it has remained unclear whether amphetamine really activates a behaviorally evident, appetitive motivational state. Following a characterization of exploratory behavior of crayfish (experiment 1), we tested whether amphetamine enhanced the exploration of surrounding cues. Since novelty-induced behavioral activation could mask the effects of drugs, a subset of experiments administered amphetamine in an environment to which crayfish had previously been habituated. Past CPP experiments utilized a single 5 mg/kg application of amphetamine, which represents a high dose.<sup>1</sup> Accordingly, we obtained a dose-response curve for systemic amphetamine infusions (experiment 2). To determine the role of head ganglia in the orchestration of different exploratory patterns, we tested for changes in extent and time course of behavioral effects following amphetamine administration into the brain rather than the general circulation (experiment 3).

## 2. Materials and methods

### 2.1. Animals

Crayfish (*Orconectus rusticus*) were wild-caught in the Portage River (near Bowling Green, OH, USA) and maintained in a community tank inside the laboratory. Prior to each experiment, intermolt individuals were isolated for a minimum of 3 days in individual plastic containers (160 mm diameter, 95 mm depth) at a 16:8 h light:dark cycle and maintained on holding trays with a continuous flow of filtered, aerated water at 20 °C. Crayfish were fed twice a week with a combination of fish, earth-worms and rabbit chow.

### 2.2. Experimental procedure

Experiment 1 characterized the behavior patterns that crayfish exhibit in novel environments. Towards this goal the types of active exploratory behavior were described and their frequencies quantified. Following 3 days of isolation, 2 experimental groups were formed. Individuals of the *control group* (N=6) were transferred to a test arena (Plexiglas aquarium (0.6×0.4×0.25 m) with a gravel substrate) and left undisturbed for a 4 h habituation period. At the end of this period, each animal was captured and immediately placed back into the experimental arena. Individuals were videotaped for 40 min with a digital camcorder (XL1, Canon, Japan) mounted above the aquarium. Crayfish of the *experimental group* (N=6) were placed in the arena and videotaped immediately after transfer (without waiting for the 4 h habituation period). Any consistent differences in behavior between the two groups are interpreted as an unconditioned response to novelty.

Experiments 2 and 3 were designed to monitor changes in these exploratory behavioral effects that result from infusion of different

doses of D-amphetamine. One day prior to the experiment, a cannula was implanted either into the pericardial sinus (experiment 2) or the head ganglion (experiment 3). On the test day, 0.5 ml of deactivated, fine-bore, fused silica needle material (Agilent, i.d.=100 μm) was connected to the cannulated crayfish with Tygon microbore tubing (Fisher Scientific, i.d.=250 μm). The infusion cannula was connected to a microdialysis pump (CMA/102) via to a microdialysis swivel mounted above the aquarium. The cannula was primed to fill its void volume to insure that drug (or saline) was delivered immediately when the pump was turned on. Crayfish of different *dose-response groups* were placed in a white Plexiglas aquarium (0.6×0.4×0.25 m) with a gravel substrate and left undisturbed for 4 h. Following the habituation phase, crayfish behavior was videotaped for 40 min. A range of doses of D-amphetamine sulfate (FW: 368.5; Sigma, St. Louis: A 5880) were administered systemically into the pericardial sinus of crayfish in experiment 2 (0.1, 0.5, 1, and 5 mg/kg of body weight), and directly into the brain in experiment 3 (at 0.1, 0.5, and 1 mg/kg). A vehicle-injected (125 mM saline) group served as control in both experiments. The total injection volume for experiment 2 was adjusted to not exceed 1/50 of the estimated hemolymph volume for each animal (Panksepp and Huber, 2004). In experiment 3, the total injection volume was 0.5 μl for all animals. Crayfish (10.9–31.5 g) were randomly assigned to 5 groups in experiment 2 (saline control [C], amphetamine 0.1 mg/kg [A01], 0.5 mg/kg [A05], 1 mg/kg [A1], and 5 mg/kg [A5]) and to 4 groups in experiment 3 (saline control [C], amphetamine 0.1 mg/kg [A01], 0.5 mg/kg [A05], and 1 mg/kg [A1]). The 5 mg/kg dose of amphetamine was excluded for the brain infusion study as it produced repetitive tail-flips and convulsions. Each group contained 7 animals at the outset, but a few individuals had to be excluded due to death from surgery, insufficient evidence for effective substance delivery, or lack of staining (see below). A total of 4–7 valid individuals remained in each treatment group.

### 2.3. Surgery

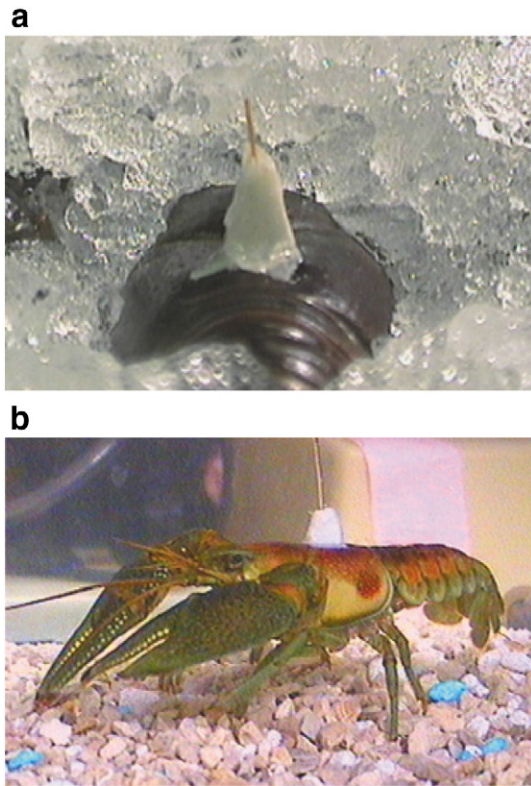
Animals were anesthetized in crushed ice for 20 min. In experiment 2, a deactivated, fine-bore, fused silica cannula (Agilent, i.d.=250 μm) was implanted into the pericardial sinus (allowing 3 mm to extend into the sinus) and secured with cyanoacrylate (Fig. 1). All animals were allowed to recover overnight within their holding containers. A successful cannula implant was confirmed via behavioral consequences of a major cocaine injection (20–60 μg) following the conclusion of the experiment. Inclusion in this study was conditional on a strong reaction to this treatment.

In experiment 3, 15 mm of deactivated, fine-bore, fused silica was implanted into the brain. Crayfish were anesthetized and mounted on a stereotaxic frame (David Kopf Instruments, Tujunga, CA). The coordinates were calculated from a conjunction point (P) between three lines evident in the head exoskeleton. The coordinates were as follows (in mm): –1 antero-posterior, ±0 lateral and –3 ventral. After insertion, the fused silica cannula was fixed in place with cyanoacrylate. Animals were allowed to recover overnight in their holding containers. Successful placement of cannulae was confirmed via methylene blue staining. At the end of the experiments, individuals were injected with the dye and decapitated. The brains were dissected, stored in 4% paraformaldehyde for 24 h, and then placed in 20% sucrose solution before sectioning. Only animals with unambiguous methylene blue stained brains were used in statistical analyses.

### 2.4. Behavioral analysis

Initial observations, revealed several conspicuous behavioral patterns during a crayfish's exposure to a novel Plexiglas aquarium. A number of behavioral differences distinguished the crayfish that were acclimated from those that were not, including locomotion (all forms of walking), antenna movements (whipping antennae back and

<sup>1</sup> It was reported that at such high dose, amphetamine and cocaine reduced locomotor activity, as the animals spent most of the time in a corner of the arena with compulsive movements of their antennae (Panksepp and Huber, 2004). Such behavior may represent an equivalent to the drug-induced, stereotyped behavior commonly seen in mammals following high doses of psychostimulants.



**Fig. 1.** (a) Crayfish are chilled in ice. A small hole is drilled through the dorsal carapace within the caudal third of the pericard, a few millimeters to the right of the midline to avoid damaging the underlying heart. Ten centimeters of fused silica tubing (J&W Scientific, #160-2644) are cut, pre-rinsed with 125 mM NaCl, and fitted with a small piece of paper towel 3 mm from one end to restrict how far the tube will protrude into the sinus. (b) The tubing is then placed into the hole and secured with small drops of cyanoacrylate and pieces of paper towel. Deactivated needle material (J&W Scientific, #160-1010) is cut to 0.5 m length with both ends reinforced with 1 cm of .250 mm outer diam., fused silica material (J&W Scientific, #160-2255). A small piece of 0.28 mm ID polyethylene tubing connects this tubing to a blunt-tipped, hypodermic needle (26 g × 1/2") attached to a 1 ml syringe. After pre-rinsing the system with NaCl, the flexible cannula is connected with PE tubing material to the short piece of stiff, fused silica already attached to the animal.

forth), rearing (standing on its last pair of walking legs, reaching upwards along the wall), site building (excavating a depression in the gravel), grooming (cleaning and maintaining parts of the body), and tail-flip (escape behavior). The pharmacological part of this study quantified changes in these behaviors as a result of amphetamine infusion. The 40 min test session was recorded on video and the duration of time spent in each (mutually exclusive) behavior was quantified with the aid of JWatcher software (<http://www.jwatcher.ucla.edu/>).

### 2.5. Statistical analyses

Statistical analyses were applied to relative frequencies of each behavioral category and analyzed using one-way ANOVAs. The independent variables were experience (habituated or novel) for experiment 1, treatment dose (C, A01, A05, A1 and A5) for experiment 2, and treatment dose (C, A01, A05, and A1) for experiment 3. All post-hoc comparisons used Duncan's test with the level of significance ( $p$ ) set at <0.05.

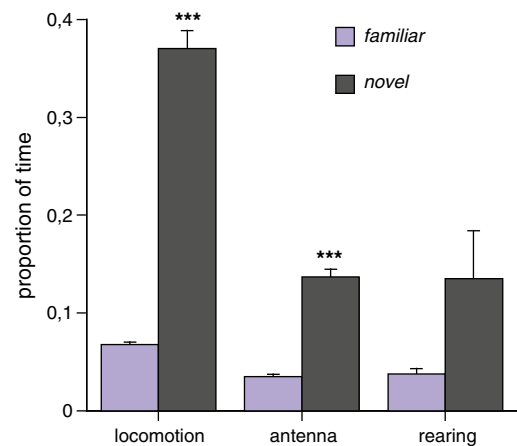
## 3. Results

In experiment 1, one-way ANOVA revealed significant effects of an acclimation period with a reduction in locomotion ( $F[1,9] = 183.142$ ;  $P < 0.001$ ), and antenna movements ( $F[1,9] = 49.429$ ;  $P < 0.001$ ). The exposure to a novel environment increased the proportion of time

spent in the two active exploratory behaviors (Fig. 2) with a robust increase in locomotion. Novelty did not affect other behavioral patterns such as site building ( $F[1,9] = 0.113$ ;  $P > 0.05$  ns), or grooming ( $F[1,9] = 0.179$ ;  $P > 0.05$  ns). Tailflips were rare in both groups, precluding a statistical analysis.

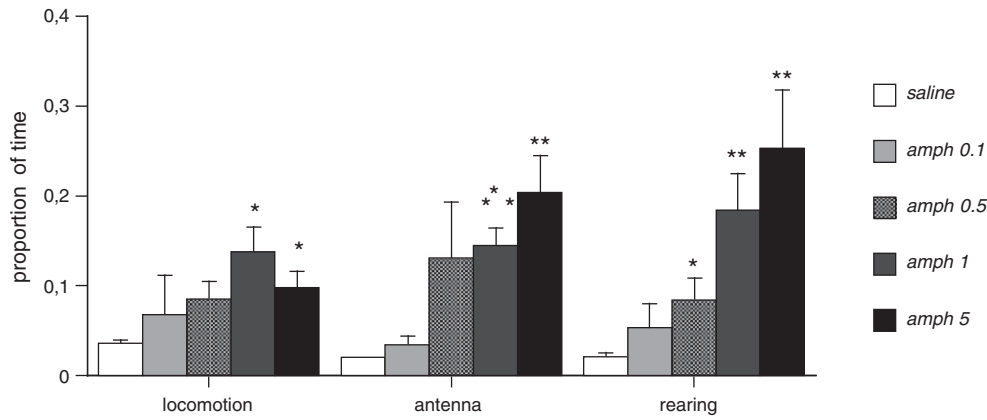
In experiment 2, one-way ANOVAs revealed significant effects of amphetamine treatment for all active exploratory behaviors, including antenna movements ( $F[4,20] = 3.816$ ;  $P < 0.05$ ), rearing ( $F[4,20] = 5.435$ ;  $P < 0.01$ ), and locomotion ( $F[4,20] = 12.390$ ;  $P < 0.001$ ). These effects were dose-dependent. Compared with saline, post-hoc analysis identified significant differences in rearing for doses of 0.5 mg/kg ( $P < 0.05$ ), 1 mg/kg ( $P < 0.001$ ), and 5 mg/kg ( $P < 0.01$ ). Amphetamine significantly enhanced antenna movements and locomotion at doses of 1 mg/kg ( $P < 0.01$ ), and 5 mg/kg ( $P < 0.01$ ). In sum, systemic amphetamine injections consistently increased the amount of time crayfish pursued active exploration of the observation arena in a dose-dependent fashion (Fig. 3). Compared to the 1 mg/kg dose, 5 mg/kg amphetamine increased rearing and decreased locomotion, suggesting a certain level of motivational competition between these behaviors at high arousal levels. One-way ANOVA revealed significant effects of amphetamine in tail flip behavior ( $F[4,20] = 3.276$ ;  $P < 0.05$ ), with post-hoc analysis showing a significant increase at the 5 mg/kg dose ( $P < 0.01$ ) (data not shown). Systemic amphetamine did not change the frequencies of site building ( $F[4,20] = 0.115$ ;  $P > 0.05$  ns) or grooming ( $F[4,20] = 0.048$ ;  $P > 0.05$  ns).

In experiment 3, one-way ANOVAs revealed significant effects of amphetamine in locomotion ( $F[3,16] = 9.316$ ;  $P < 0.001$ ), antenna movements ( $F[3,16] = 17.082$ ;  $P < 0.001$ ), and, to a lesser degree, for rearing (Fig. 4). Post-hoc analysis demonstrated a significant increase in locomotion for amphetamine at the 0.5 mg ( $P < 0.05$ ) and 1.0 mg dose ( $P < 0.001$ ). Post-hoc analysis showed significant differences in antenna movements for crayfish receiving 0.1 mg ( $P < 0.05$ ), 0.5 ( $P < 0.01$ ) and 1.0 mg amphetamine ( $P < 0.001$ ) compared to saline. In rearing behavior, only animals receiving the 0.5 mg amphetamine dose ( $P < 0.05$ ) differed from saline controls. Compared to 0.5 mg/kg, a 1 mg/kg dose increased locomotion and antenna movements, while reducing rearing. This finding supports the idea of a possible motivational competition between locomotion and rearing. When injected into the brain, amphetamine did not significantly influence tail flips ( $F[3,16] = 1.018$ ;  $P = 0.412$ ), grooming ( $F[3,16] = 0.753$ ;  $P = 0.537$ ) or site building behaviors ( $F[3,16] = 0.901$ ;  $P = 0.463$ ). Direct application of amphetamine to the brain selectively enhanced exploratory behaviors in a dose-dependent fashion as it did with



**Fig. 2.** Effects of environmental conditions (familiar vs novel) on crayfish exploratory behaviors during a 40 min period. Results are expressed as the mean percentage of time spent performing each behavioral category. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared to behavior in the familiar environment.





**Fig. 3.** Effects of different doses of systemic amphetamine injections on crayfish exploratory behaviors during a 40 min period. Results are expressed as the mean percentage of time spent in each behavioral category. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared to saline.

systemic infusions—a primary site of action in the supraesophageal ganglion is thus suggested.

#### 4. Discussion

Collectively, these findings demonstrate that D-amphetamine enhances exploration in crayfish, with increased locomotion and exploratory sampling of the environment. Active exploration, as highlighted by patterns for locomotion and antenna movements, is enhanced in crayfish which are placed into a novel environment, compared to others who had already habituated to the test arena. Novel stimuli appear to directly influence brain networks that facilitate exploratory behaviors that are normally used in pursuit of natural rewards. It is likely that these appetitive motivational states may be recruited by psychostimulants such as amphetamine.

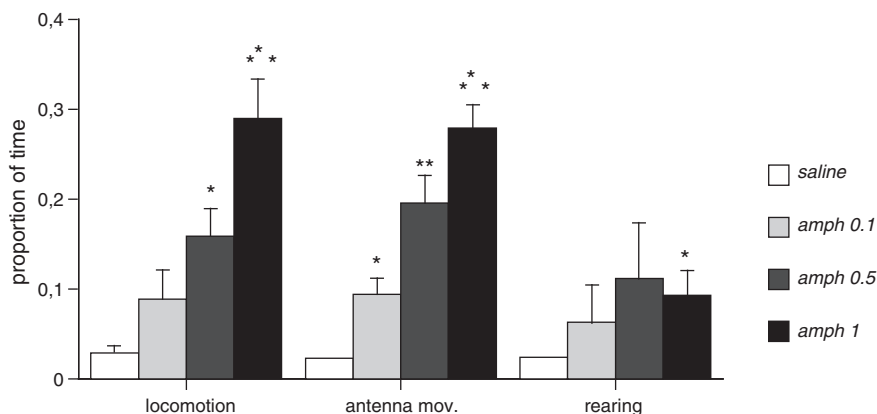
Crayfish, habituated to their environments, tended to settle into a location along an edge or corner. Systemic administration of amphetamine induced strong arousal, locomotion, and increased exploration of the test arena. The most effective dose of amphetamine (1 mg/kg) enhanced locomotion by 383%, antenna movements by 725% and rearing by 876%. Such behavioral responses to amphetamine suggest increased brain monoaminergic transmission (Fleckenstein et al., 2007). It is possible that amphetamine simply causes a generalized arousal state, and that the observed behaviors are merely the consequence of such activation. However, other behaviors transiently exhibited in habituated environments (e.g., activity directed towards site building or grooming) were unaffected by amphetamine,

suggesting selective effects towards those behavioral patterns that are specifically associated with exploration.

Systemic administration of amphetamine at the highest dose (5 mg/kg) also increased the occurrence of tail flips. This is not surprising since monoamines play a modulatory role in the escape circuit of crayfishes (Glanzman and Krasne, 1983) and preliminary observations in our lab have demonstrated that amphetamine facilitates escape patterns. However, tail flip was stimulated when amphetamine was injected in an acute treatment of less than 5 min. In contrast, injection utilizing longer time-frames of around 20 min resulted in considerably fewer tail flips, with minimized escape, and amplified SEEKING.

Administration of amphetamine directly into the brain was more effective than peripheral drug application in enhancing the above behaviors; for instance, central application of 1 mg/kg amphetamine increased locomotion by +1000% and antenna movements by +1210% but produced only modest effects on rearing (+385%). Moreover, other behavioral patterns such as tail flips, grooming, and nest building were unaffected by amphetamine administration directly to the brain. Since locomotor activity and antenna movements are largely exploratory, we surmise that the aroused appetitive states were mainly related to the effect of amphetamine within the crayfish head ganglion. Enhanced rearing, which in contrast is mainly directed towards escaping from the arena, may have been mediated by more distal ganglionic actions of the drug. Site-specific, pharmacological experiments are needed to further pinpoint the precise anatomical sites of such behavioral effects.

Exploratory behaviors in crayfish are driven by tactile and olfactory information (Kraus-Epley and Moore, 2002; McMahon



**Fig. 4.** Effects of different doses of direct brain amphetamine injections on crayfish exploratory behaviors during a 40 min period. Results are expressed as the mean percentage of time spent in each behavioral category. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared to saline.

et al., 2005; Patullo and Macmillan, 2006), detected primarily via antennae and antennules, and conveyed mainly to the olfactory lobe of the brain (Mellon, 2000; Sullivan and Beltz, 2005). Modulated by serotonin and dopamine transmission (Sandeman and Sandeman, 1987; Sandeman et al., 1995; Schmidt, 1997), the olfactory lobe of crayfish represents a site of action of amphetamine, and perhaps other drugs as well. The perception of the external environment is not a passive process, but is characterized by active movements as animals orient their sensory organs towards specific sources of stimulation, and move in specific directions to localize and interact with objects of interest. Locomotion and antenna tactile exploration act in concert with other sensory information to produce whole-body adaptive responses. For instance, navigation of *C. elegans* in resource-rich environments is controlled by central neural integrators that span primary sensory neurons for olfaction and taste, interneurons, and motor neurons (Gray et al., 2005). The presence of similar amphetamine-sensitive circuits within the crayfish brain suggests the presence of evolutionarily conserved processes that promote exploratory and approach behavior patterns through increased incentive salience of surrounding stimuli or enhance exploratory motor patterns—a distinction that needs further investigation.

In sum, our data are consistent with rewarding properties of amphetamine in crayfish as determined by CPP procedures (Panksepp and Huber, 2004), and such reward components may arise from the activation of a disposition for exploration and approach within the crayfish brain. The brain's integrative control over such adaptive responses may promote the organism's ability to search for favorable, life-supporting environmental conditions. A comprehensive understanding of the causation of this behavior beyond the intervening level of motivations requires that it ultimately is mapped onto their respective proximate mechanism. The neurochemistries and functional neuroanatomies of reward seeking brain circuits in crayfish remain to be detailed, but monoamines appear to play an important role. Monoamine systems are attractive candidates as they modify neural function at multiple levels and may thereby bring about coordinated responses to environmental perturbations. In this manner, amines are thought to alter the activity of neural decision-making centers (Nader et al., 1997). Rather than produce behavior *per se*, these substances appear to tune ongoing activity and, in a given context, promote the occurrence of adaptive behaviors (e.g. feeding, flight, fight, or mating) over contra-adaptive ones (Kravitz, 1988). Among the monoaminergic systems present in the crayfish's head ganglion (dopamine, serotonin, and octopamine), research on both invertebrates and mammals now suggest that exploration and approach may specifically depend on dopaminergic function (Hills et al., 2004; Alcaro, et al., 2007).

In mammals, the mesolimbic dopamine system has been indicated as a key component of complex appetitive-motivational neural circuits (Alcaro et al., 2007) which, to highlight its primary-process (i.e., instinctual) status, has been labeled the SEEKING emotional system in mammals (Panksepp, 1981, 1998). The primal function of this system is to sustain exploratory approach behaviors that optimize encounters with a large variety of conditions needed for survival. Certain drugs prove addictive when they activate this pro-survival mechanism, probably because the underlying action pattern itself is rewarding. Through learning, this system facilitates processing of the acquired affective incentive value for cues associated with natural and drug rewards (Di Chiara and Bassareo, 2007). The present work supports the conclusion that ancestral forms of a similar functional circuit exists in invertebrate brains. Further work towards characterizing this circuit, especially the role of dopamine and other neurochemicals within brain systems for cardinal survival functions, will allow us to better understand the relationships between ancestral appetitive motivational mechanisms and the fundamental brain sources of drug addiction.

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