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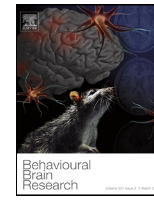

Research report

# Exploratory behavior and withdrawal signs in Crayfish: Chronic central morphine injections and termination effects

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## HIGHLIGHTS

- Novel stimuli directly augment exploratory behaviors in crayfish.
- Morphine increase locomotion and exploration of the environment.
- Termination of morphine injections results in withdrawal signs in crayfish.

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## ABSTRACT

Functional and evolutionary conservation of neural circuits of reward seeking >is a symbol of survival. It is found in most animals from insects to humans. Exploration is a component of a wide range of drug-elicited behaviors that reflects an appetitive motivational state when animals seek natural rewards such as food, water, and shelter for survival. Not only does the characterization of exploratory behaviors indicate the specific components of appetitive motor patterns, it also reveals how exploratory behavioral patterns are implemented via increased incentive salience of environmental stimuli. The current work demonstrates that novel stimuli appear to directly augment exploration in crayfish, while injections of morphine directly into the brain of crayfish enhanced robust arousal resulting in increased locomotion and exploration of the environment. Elimination of morphine suppressed exploratory motor patterns. Crayfish displayed atypical behavioral changes evident of withdrawal-like states when saline is injected into the brain. With proven evidence of rewarding to the exposure to mammalian drugs of abuse, modularly organized and experimentally accessible nervous system makes crayfish exceptionally suitable for characterizing the central workings of addiction at its key behavioral and neuroanatomic locations.



## 1. Introduction

Drugs such as amphetamine, cocaine and morphine are known to promote unconditioned behavioral responses and increased locomotion to enhance exploration and approach behaviors. Animals display exploration and approach dispositions in their natural environment to signify an appetitive motivational state when they seek natural rewards [1,2]. Mammalian drugs of abuse are able to seize control and enhance exploration and approach behaviors when the brain fails to distinguish whether specific reward circuits were activated by adaptive natural rewards or falsely triggered by a class of addictive psychostimulant substances [3]. Several lines of evidence indicate that the rewarding properties of drugs of abuse in mammals originate from the stimulation of the neural processes involved in the activation of the appetitive states. These states include exploration and approach dispositions which represent major components of the seeking system [4]. Crayfish, an invertebrate model of drug addiction exhibit similar dispositions during a search for food, shelter and protection in the natural environment. The crayfish model represents an invertebrate model that has been used in previous studies [5–8] to identify and manipulate the neural circuits responsible for sensory input to motor output (exploration), and to characterize how drugs of abuse may affect such activities in an invertebrate model of drug addiction. With a comparatively simple nervous system that has neuromodulatory organization amenable to genetic manipulations, the crayfish system has emerged as a useful animal model of addiction. The worth of crayfish as a model system for studies of addiction was not previously recognized because a drug-reward phenomenon had not been documented. The demonstration that crayfish natural reward

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was surprisingly sensitive to human drugs [5–13] made the crayfish model particularly suited to characterize specific behaviors that are relevant in reward seeking. Representing an invertebrate animal model with proven evidence of rewarding to the exposure to mammalian drugs of abuse, modularly organized and experimentally accessible nervous system makes crayfish exceptionally suitable for characterizing the central workings of addiction at its key neuroanatomic locations. The current study is to advance our previous studies by characterizing motor reactions of morphine-withdrawn or abstinence in crayfish including components of exploratory behavioral patterns.

In mammals, abstinence from repeated morphine intake expresses itself when administration of the drug is terminated. Abstinence is thought to be characterized by different behavioral signs that follow a distinctive temporal or long term course. The nature of the exact behavioral changes that constitute a withdrawal syndrome from prolonged or acute morphine treatments have been investigated extensively in mammals [14–18]. These studies identified piloerection, chills, severe diarrhea, nausea, vomiting, diaphoresis, myoclonus, and mydriasis as classical symptoms of withdrawal in mammals. Furthermore, naloxone-precipitated withdrawal produced defensive and escape behavior in awake rats [19]. When self-administration of heroin by rats was terminated after several weeks, ongoing food-reinforced behavior and locomotion was disrupted for several days [20].In addition, a withdrawal from continuous administration of morphine attenuates the increase in thermal sensitivity seen in morphine -treated mice [21]. This finding indicates that environmental factors can alter withdrawal behaviors following the termination of chronic drug administration. Morphine withdrawal is generally known to facilitate the discrimination of different stimuli. Rats trained to discriminate naloxone from saline using a drug discrimination test, successfully discriminated this stimulus during withdrawal from a 7-day regimen of morphine [22]. Similarly, rats displayed more behavioral morphine withdrawal symptoms in the absence of the drug, when they are placed in the previously drug-paired environment than when they are placed in an alternative environment [16]. Comparative conclusions were made when protracted morphine administration withdrawal led to the observed reduced emotional signs in rats conditioned to low saccharin compared to rats conditioned for high saccharin intake [23]. The result indicates that withdrawal can modulate reward seeking of both drug and non-drug reinforcers in mammalian models of drug addiction following behavioral sensitization.

Few studies have also documented evidence of withdrawal in invertebrates. For instance, abstinence-induced or precipitated withdrawal-like behaviors have been shown in planarians [24,25]. In another study, nociception attenuates methamphetamine abstinence-induced withdrawal-like behavior in planarians [25], while planarians display screw-like hyperkinesia following morphine withdrawal. When a morphine antagonist (naloxone) was injected into a snail (*Megalobulimus* sp.), the snail displayed a stereotyped and reproducible avoidance behavior that reflect withdrawal signs [26]. Whether there is evidence of withdrawal signs in crayfish -an invertebrate animal model with proven evidence of rewarding to the exposure to mammalian drugs of abuse is yet to be determined. The objective of the current study is (i) to characterize morphine-induced exploratory behavior, (ii) determine whether abstinence from repeated morphine-use in crayfish can alter exploratory behavior and (iii) manifest itself in unique behaviorally evident, withdrawal-like states when morphine injections are terminated. The first set of experiments characterized exploratory behavioral patterns in crayfish. The second experiments examined the consequences of brain morphine injections on crayfish exploratory behaviors in a dose-dependent manner. The third experiment identifies and quantifies atypical behavioral changes evident of withdrawal-like states when morphine injection is terminated, and saline is injected into the brain. This paper reports a robust set of characteristic changes in crayfish behavior that resulted from termination of morphine, a mammalian drug of abuse.

## 2. Materials and methods

### 2.1. Experimental procedure

#### 2.1.1. Animals

Fourteen male intermolt male crayfish (*Orconectes rusticus*) with complete and intact appendages purchased from Carolina Biological Supply Company were used for this study. In the laboratory, the animals were maintained in a big tank of water (400 gallons) that is freshly aerated and flows through holding trays. Once in the laboratory, animals were isolated in individual plastic containers and maintained in flow-through holding trays that received freshly filtered/aerated water at $20 \pm 1\,^{\circ}C$. Crayfish were fed 1–2 times per week with tuna fish, earthworms or rabbit chow, and housed under a 16:8 h light/dark cycle.

### 2.2. Experimental procedure

#### 2.2.1. Spatial characteristics of crayfish in a novel environment

*2.2.1.1. Behavioral experiment 1.* The methods used by Alcaro et al. [9] were adapted to characterize exploratory behavioral patterns that crayfish exhibited in a novel environment. Two experimental groups were formed. Group 1 ($n = 9$) explored the spatial activities of crayfish (body weights between 13.5 and 30.3 g) inside the test aquarium (with gravel substrate), to determine the behavioral patterns that crayfish exhibited in a novel environment. Group 2 ($n = 9$) explored the behavioral patterns of crayfish inside a test aquarium with a plain background. The different components of active exploratory behavior of crayfish were characterized in both groups. Crayfish were isolated for two days from the big tank of water where they were usually kept. Isolated animals ($n = 9$) were transferred into the novel environment or aquarium with plain background. Strip lamps with 20 W florescent bulbs were mounted at the sides of the experimental arena. A digital Carl Zeiss Sony DCR-VX1000-NTSC camera with 40× optical zooming was placed above the aquarium to cover the entire aquarium providing an area profile view. Aerated water was continuously passed through the aquarium. On day 1, animals were left undisturbed to acclimatize for 2 hrs in each experimental arena. Following the 2 h habituation time, each animal in group 1 was removed and instantaneously returned into the experimental arena to characterize exploratory behavioral changes in a novel environment. This experiment was repeated for animals in group 2 on the plain background. To determine whether there are consistent differences in behaviors of crayfish between the two environments (plain and gravel), we reversed the order of testing in the second day of the experiment. Precisely, on day 2, animals in group 1 were tested in the aquarium with plain background, while animals in group 2 were tested in the aquarium with a gravel substrate on the floor. Spatial activities of individual animals were videotaped each for 40 min. Consistent exploratory behavioral patterns between the two contrasting environments were characterized and considered as unconditioned responses to novelty.

### 2.3. Surgical protocol for the implantation of cannula into the brain of Crayfish

Animal were anesthetized in crushed ice for about 20 min. Successful anesthesia was evident when the appendages were not moving in the crushed ice. Anesthetized animals were mounted